

Natural killer cells suppress cardiac inflammation and fibrosis by inhibiting eosinophil
accumulation

By

SuFey Ong

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ABSTRACT

Myocarditis is the inflammation of the cardiac muscle and a leading cause of sudden cardiac failure. Previously, our lab has shown that natural killer (NK) cells suppress group B coxsackievirus (CVB) and mouse cytomegalovirus (MCMV)-induced viral myocarditis severity by minimizing virus replication. We demonstrate that the protective capabilities of NK cells in myocarditis extend beyond controlling viral infection into directly controlling autoimmune-mediated inflammation. Experimental autoimmune myocarditis (EAM) is instigated in BALB/c mice by immunization with cardiac myosin peptide. NK cells accumulated in the heart through EAM, secreted IFN γ , perforin, and granzyme-B, and had upregulated levels of activation receptors compared to peripheral NK cells. Wildtype (WT) mice undergoing EAM showed significantly greater disease severity when NK cells were depleted using anti-asialo GM1 antibodies. This was accompanied by increased cardiac inflammation, collagen deposition, and a 10-fold increase in the proportion of infiltrating eosinophils. This increased influx of eosinophils was required for the effects of NK depletion in WT mice, indicating that NK cells control disease severity through the suppression of eosinophil infiltration. NK cells had the ability to control eosinophil-related chemokine expression from primary cardiac fibroblasts (CF) – levels of eotaxin 1 (CCL11), eotaxin 2 (CCL24), CXCL9, and CXCL10 were regulated by NK cells both *in vivo* and *in vitro*. Altogether, we show a new pathway of eosinophilic regulation through interactions with NK cells and open a new line of therapeutic research in the treatment of myocarditis.

PREFACE

Persistence, strategy, and good fortune are three major variables in the determination of success. While I cannot always speak to the former two elements, I have always had the incredible luck of being surrounded by amazing personal and academic communities. Without the efforts and support of the many people (below being an incomplete list) I have depended on and learned from in the past twenty-six years, my well of potential would be more of a puddle.

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CATALOGUE OF COMMONLY USED ABBREVIATIONS

AHA	American Heart Association
ASGM-1	Anti-asialo GM-1 antibody
CBV	Group B coxsackievirus
CF	Cardiac fibroblasts
DCM	Dilated cardiomyopathy
DCMI	Inflammatory dilated cardiomyopathy
EAE	Experimental autoimmune encephalomyelitis
EAM	Experimental autoimmune myocarditis
EMB	Endomyocardial biopsy
HSC	Hematopoietic stem cell
IL	Interleukin
LV	Left ventricle
MCMV	Murine cytomegalovirus
NK	Natural killer
OVA	Ovalbumin peptide
TLR	Toll-like receptor

CHAPTER I: INTRODUCTION TO MYOCARDITIS AND DILATED CARDIOMYOPATHY

Myocarditis: Definition and Diagnosis

Myocarditis is the inflammation of the cardiac muscle¹. Histopathologically, myocarditis is defined by the Dallas criteria to be the presence of inflammatory infiltrate associated with myocyte necrosis in the absence of ischemia²⁻⁴. Presence of cardiomyocytes necrosis with an inflammatory cellular infiltrate is characteristic for active myocarditis⁵. Several different subtypes of myocarditis exist depending on histological assessment. If there is inflammation in the absence of myocyte death, the disease is defined as borderline myocarditis. In the presence to necrosis, the major infiltrating cell-type will distinguish lymphocytic, giant cell, and eosinophilic myocarditis, though other cell types may also be present (Table 1)⁶.

Histological determination of disease requires tissue obtained by endomyocardial biopsy (EMB) or post-mortem autopsy. Immunohistochemistry may further delineate inflammatory cell populations based on surface markers such as MHCII and CD3⁷⁻⁹. EMB is widely used in Europe, but its use remains limited in the United States^{10,11}. In the US, the view is that limited sampling results in EMB-based myocarditis diagnosis being prone to low sensitivity and difficulties in establishing a prognosis¹². Perceived risks of patient endangerment, extensive costs, and limited accessibility to expert pathological interpretations underline the need for improved tools in the early diagnosis of myocarditis¹⁰. However, the European Society of Cardiology strongly supports EMB for all clinically suspected myocarditis patients. The complication rate is low and most importantly only EMB can confirm the myocarditis infection status, necessary for determining the patient treatment.

Clinical symptoms of myocarditis may range from mild chest paints to cardiac

shock and ventricular arrhythmias¹². Clinical myocarditis can be divided based on clinical and pathological criteria as fulminant, sub-acute, chronic active, and chronic persistent subtypes^{13,14}. Fulminant myocarditis is defined as active myocarditis accompanied by acute, severe cardiac failure¹⁵. Immediate intervention may be necessary for cardiac survival¹⁶. If diagnosed quickly and treated aggressively, however, the large majority of fulminant myocarditis patients will make a full recovery. Non-fulminant cases, despite not requiring immediate intervention, appear to have decreased long-term survival¹⁷.

Several methods have been undertaken to improve diagnosis of myocarditis in a non-invasive manner. Studies have examined markers of cardiac injury, such as cardiac troponin I (cTnI)¹⁸, myosin or cTnI antibodies^{19,20}, and creatine-kinase MB²¹, but these signs do not distinguish between different causes of cardiac myocyte injury and do not alone improve the determination of future therapy^{22,23}.

Echocardiography, a first-line tool in the identification of cardiac abnormalities, is of limited use in the positive diagnosis of myocarditis. Myocarditis patients do not display any specific echocardiographic characteristics. While some parameters such as segmental wall abnormalities, LV wall thickness or ventricular thrombi showed some association with myocarditis, they are indistinguishable from other cardiac conditions such as myocardial infarction²⁴. Echocardiography is an excellent method for ruling out other causes of heart failure and to monitor patient progress, but cannot be relied on a sole diagnostic tool^{15,25,26}.

Electrocardiograms (EKG) may also be problematic in the positive diagnosis myocarditis^{25,27}. Acute myocarditis patients may display non-specific ST-segment and T-wave abnormalities or present with elevated ST-segments that directly mirror an acute

myocardial infarction^{28,29}. In one study of 42 histologically proven myocarditis patients, 58% had normal P-waves, 49% had normal QRS-complexes, and 64.5% had no repolarization abnormalities. In summary, the predictive value of EKG was low, although all myocarditis patients displayed at least abnormality among the parameters measured. Of these, abnormal QRS complexes and left bundle branch block (LBBB) were most strongly correlated with poor clinical outcomes³⁰.

Cardiac magnetic resonance imaging (MRI) has shown great promise among non-invasive techniques being used for diagnosis of acute myocarditis, especially in the earliest stages of disease^{4,31}. T2-weighted images may show the presence of inflammation-associated edema in the myocardial tissue³². Myocardial global relative enhancement (gRE) studies to examine myocardial hyperemia and increased capillary permeability have sensitivities between 63-85%³³. Furthermore, the use of cardiac MRI-guided EMB to facilitate sampling of the abnormal regions of the LV is a powerful tool in improving the diagnosis of myocarditis³⁴.

In conclusion, EMB remains the gold standard for myocarditis diagnosis. While the inclusion of multiple non-invasive tests such as serum inflammatory markers and cardiac MRI do not replace EMB, they serve as complement during the diagnostic process of myocarditis and subsequent patient monitoring.

Myocarditis Etiology

Multiple agents are associated with the induction of clinical myocarditis (Table 2)³⁵. However, in most cases, no specific toxic agent or active pathogen can be

identified³⁶. The causative event may have occurred well before the onset of clinical symptoms, leaving no trace in the patient. Difficulties in determining the etiology of myocarditis results in a large majority of cases being identified as idiopathic¹².

Viruses are the most commonly identified causes of myocarditis³⁷. Parvovirus B19 is the most commonly isolated viral pathogen in Europe, a recent shift from group B coxsackievirus (CVB) and adenovirus^{9,38}. Studies done with hepatitis C found strong correlations between HCV antibodies and RNA in proven myocarditis patients³⁹. Other associated viral pathogens include HIV, Epstein-Barr virus, cytomegalovirus (CMV), and herpesvirus⁴⁰. There is, however, little evidence for an association between levels of virus and clinical severity of myocarditis⁴¹⁻⁴⁴.

There is also increasing recognition of the myocarditis brought on by infection with *Trypanosoma cruzi*, a growing public health concern as the disease spreads north from Central and South America. Infection with *T. cruzi* results in Chagas disease. This flagellated parasite enters the bloodstream through the fecal matter of their vector, the triatomine bug. Upon scratching at the bite site, the fecal matter is smeared into the broken skin and the flagellates invade their new host⁴⁵. While the course of disease is unpredictable, the acute phase is associated with transient, febrile myocarditis. Etiologically, it is thought to be associated with cross-reactions of the immune system between *T. cruzi* proteins and cardiac muscle⁴⁶. Afterwards, 30% of patients will gradually develop severe chronic myocarditis despite a scarcity of parasites found in the heart⁴⁷. Largely found in South America, cases and infected vectors have been found in the southern United States and there have been several reports of infection through transfusions from imported blood^{48,49}.

Hypersensitivity reactions to drugs also constitute a significant percentage of myocarditis patients^{50,51}. The list of drugs associated with myocarditis vary greatly, with Table 2 providing a limited list, and can occur in patients where they were previously well-tolerated⁵². Often, this hypersensitivity reaction responds to the withdrawal of the offending drug agent and the application of corticosteroids⁵²⁻⁵⁴.

Autoimmune mechanisms may play a role in the development of myocarditis. Viral invasion in the heart results acute cardiac damage and the release of multiple self-antigens into an inflammatory environment⁴³. Also, individuals with well-established autoimmune disorders such as systemic lupus erythematosus (SLE), hyper IgE (Job's) syndrome, celiac disease, and multiple sclerosis can present with secondary myocarditis⁵⁵⁻⁵⁸. 41-56% of myocarditis patients produce autoantibodies against cardiac proteins, most commonly against cardiac myosin heavy chain, and additional proteins such as cTnI and skeletal myosin may also be targeted^{35,59,60}. These cardiac autoantibodies are correlated with reduced LV function in chronic myocarditis, though whether they are directly responsible is unknown^{61,62}. Additionally, CD4⁺ cells in the blood of myocarditis patients proliferate upon stimulation with cardiac myosin *in vitro*, suggesting the development of an anti-cardiac humoral immune response⁶³.

Myocarditis: Treatment

Management of clinical myocarditis is dependent on the patient presentation, suspected etiology, and disease stage. In cases of fulminant myocarditis presentation with cardiac shock and severe ventricular dysfunction, patients should first be managed in

intensive care units with hemodynamic monitoring³⁵. Some cases may require ventricular assist devices or extracorporeal membrane oxygenation (ECMO)^{64,65}. In stable patients and those presenting with non-fulminant myocarditis, combination treatments are given, aimed at decreasing cardiac load and improving function in order to limit chances of a cardiopulmonary event. Suggested treatments include diuretics, to common to prevent fluid overload and angiotensin-converting enzyme (ACE) inhibitors, which may limit maladaptive chronic remodeling if treatment is begun in the early stages of disease regardless of etiology^{5,35,66,67}.

Additionally, the use of beta-blockers results in increased ventricular function and better clinical outcomes in non-fulminant myocarditis^{4,35,68,69}. Despite this treatment and the recommended avoidance of exercise, patients may continue to develop persistent heart failure where treatments with aldosterone antagonists may be of benefit. Their mechanism of action is not well-defined, but may reduce cardiac remodeling through the suppression of fibrosis. These drugs resulted in a reduction of hospital admissions and increased survival in heart failure patients^{5,35,66,67}.

Current recommended therapies are aimed only at improving cardiac function as there are no effective specific treatments for myocarditis. The use of interferon-beta (IFN- β) to treat viral myocarditis in some studies resulted in the elimination or reduction of viral presence from the heart, the improvement in cardiac function, and an improved 10 year survival^{35,70}. Interferon-alpha 2 (IFN- α 2) in animal models of viral myocarditis resulted in the removal of viral genome in the heart and decreased cardiac inflammation and injury⁷¹.

Immunoadsorption (IA) to remove circulating IgG antibodies and intravenous

immunoglobulin treatment (IVIG) have both been utilized in clinical trials. The latter was associated with improved LV function in heart failure trials, but has not been tried in studies of histologically proven myocarditis^{72,73}. IA, aimed at limiting autoantibody levels, has been shown to decrease myocardial inflammation, though this also was not done in for histologically proven myocarditis patients⁷⁴.

Direct immunosuppression using steroids alone or in combination with azathioprine or cyclosporine has been a target of clinical trials. In RNA virus-negative and autoantibody-positive patients with EMB-positive myocarditis, treatment with prednisone and azathioprine improved LV function and decreased LV diastolic dimensions⁷⁵. Other trials, however, that treated patients with unknown etiology did not show improvement when immunosuppressive strategies were added to symptomatic treatment plans^{35,59,60}.

Approximately half of acute myocarditis patients will resolve within the first month of disease. In the remaining patients, 25-50% will experience deteriorating cardiac function despite being monitored and treated. Within this population, a secondary phenotype emerges known as inflammatory dilated cardiomyopathy (DCMI), described in the next section.

Dilated Cardiomyopathy (DCM)

Cardiomyopathies are a large and heterogeneous group of disorders associated with the morphologically abnormal appearance and failure of myocardial performance⁷⁶. DCM is classified as a type of cardiomyopathy characterized by ventricular chamber

enlargement in the absence of hypertension or other abnormal loading conditions. Physiologically, patients may present with reduced cardiac function as seen by decreased ejection fraction due increased end-diastolic dimensions.

The age-adjusted prevalence of DCM in the United States is approximately 36 cases per 100,000 individuals⁷⁶. DCM is the third most common cause of heart failure and the most common form of cardiomyopathy. 40% of DCM cases are believed to be genetic in nature, meaning that the disease has occurred at least two closely related family members^{77,78}. Around 30 genes have been identified as associated with the disease, though most familial cases are heterogeneous with private gene abnormalities⁷⁸. It has been estimated that over 90% of familial cases have been inherited in an autosomal dominant manner and show age-dependent penetrance along with variable expression⁷⁷.

Guideline-directed medical therapy (GDMT), as approved by the American Heart Association (AHA) and the American College of Cardiology Foundation (ACCF), for DCM is first aimed at reducing pressure overload by limiting hypertension through the use of diuretics, beta-blockers, and angiotensin-targeting drugs such as ACE inhibitors or angiotensin II receptor blockers (ARBs). Patients are also recommended several behavioral changes including sodium limitation.

25% of patients with recent onset of symptomatic DCM will rapidly improve even in the absence of correct GDMT. However, after 3 months of no improvements is associated with poor prognosis long-term. Some patients benefit from a left ventricular assist device (LVAD) and an implantable prophylactic fibrillator (IPD), but heart transplant remains the only curative treatment for end-stage DCM^{79,80}. DCM patients constitute the majority of heart transplants in the United States, with DCM following

myocarditis making up 9% of all DCM-related heart transplants¹⁵.

Outcomes of DCM-related heart transplants are significantly worse among those from a primary diagnosis of myocarditis. In one study by Pietra *et al*, of the 231 children awaiting cardiac transplant, 80% received transplants during the study period with 86% being UNOS Status 1. The 1, 5, and 10 year survival rates were 92%, 80%, and 72%, respectively. The strongest indicators for negative clinical outcomes were a primary diagnosis of myocarditis, older age at transplantation, and lower LV end diastolic dimensions (LVEDD). Survival of myocarditis-based DCM patients, which were 11% of the total transplants, were 83% at 1 year and 65% at 3 years, significantly lower than non-stratified survival rates⁶⁹.

The poor clinical outcomes associated with myocarditis-based DCM raises the possibility that the etiological agent of myocarditis, whether viral or autoimmune, may persist after transplantation. This underscores the need for a specific and curative therapy for myocarditis in both chronic patients and those apparently resolved cases under going cardiac transplantation due to secondary DCM.

DCM may be primary or acquired. Primary DCM is considered genetic or familial in nature, whereas secondary DCM is associated with of an inflammatory or autoimmune disorder⁸¹. Secondary DCM is also known as inflammatory DCM (DCMI), if dilation was a result of proven cardiac inflammation or inflammation is still present in the cardiac tissue. In myocarditis-based DCMI, the inflammatory infiltrate and subsequent tissue damage begins a wound-healing cascade of events that ultimately leads to significant interstitial edema, myocyte necrosis, and replacement fibrosis and collagen deposition. In some cases, underlying low levels of viral pathogen may result in continual cardiac

myocyte damage and inflammation^{82,83}. It has been postulated that the continual cardiac myocyte injury and subsequent fibrosis is driven by an autoimmune response against myocyte-associated proteins⁶⁰.

Myocarditis and DCM: Animal Models

Animal models of myocarditis and DCM are necessary in order to better study the natural history of the disease. There are several accepted models of murine myocarditis. Viral myocarditis can be induced in susceptible mouse strains with CVB or murine cytomegalovirus (MCMV) (Figure 1). Following infection, mice develop a primary disseminated viral infection by day 4, but in surviving mice a secondary and tertiary phase disease progress occurs⁸⁴⁻⁸⁷.

Inoculation of mice with CVB, similar to MCMV, can induce a fatal acute viral myocarditis within the first week of disease with substantial damage to the cardiomyocytes and disseminated viral presence. However, the Nancy strain of CBV may be heart-passaged first in order to induce a milder disease more conducive to survival and progression to the secondary and tertiary stages⁸⁴. In the surviving mice, an acute cardiac inflammatory stage occurs from day 7 and resolves by day 14 coinciding with the peak of infiltration. After day 14, the disease resolves with a tertiary chronic low-grade inflammation following day 28 onwards in susceptible mouse strains such as BALB/c or A/J. This low grade inflammation coincides with the development of DCMI. However, the genetic resistance to the inflammatory phase in C57B/6 and B10 strains can be reduced by administration of bacterial lipopolysaccharide (LPS), interleukin (IL) 1 β or tumor necrosis factor alpha (TNF- α)^{85,87}.

Active virus can be isolated from the heart only up to day 10 of infection, with detectable RNA being found until day 14^{85,88}. Hence, the chronic phase of murine viral myocarditis is thought to be largely autoimmune-driven in nature, and suggests the possibility of the virus acting as an adjuvant. In both viral models, titers of anti-myosin IgG and IgM can be found in resistant and susceptible mice strains^{89,90}. Furthermore, the antibodies found in susceptible strains are specific for cardiac myosin and are not cross-reactive with other forms of myosin⁹⁰. Other autoantibodies found include troponin I, tropomyosin, and adenine nucleotide translocator (ANT)⁹¹. It is been shown that viral replication in the heart itself is needed to cause myocardial injury. The depletion or absence of either the CD4⁺ or CD8⁺ T-cell component results in a significant reduction of the subsequent post-viral myocarditis^{92,93}. Taken together, it is likely the viral-mediated damage of the heart begins an immune cascade resulting in the development of anti-cardiac adaptive immunity.

Myocarditis can also be induced in the absence of an active pathogen. Experimental autoimmune myocarditis (EAM) is evoked by immunization of susceptible mouse strains, which are identical to the mouse strains susceptible to viral myocarditis. Mice are subcutaneously (s.c.) injected with 100ug of either whole cardiac myosin or a strain-specific myocarditogenic peptide fragment derived from α -myosin heavy chain (Table 3) emulsified in complete Freund's adjuvant (CFA) at day 0 and day 7 of disease, along with 500ng of pertussis toxin intraperitoneally (i.p.) to further stimulate the immune system^{61,84,94}. The different peptides used to induced EAM in various strains indicates that different epitopes of cardiac myosin are targeted by the immune system, as strains are generally not reactive to other peptides. A/J mice immunized with cardiac

troponin I instead of cardiac myosin develop similar disease⁹⁵. Importantly, EAM mimics the clinical myocarditis-based progression to a secondary disease phase, DCMI, in nearly all mice immunized.

EAM is marked by a priming phase from day 0 to 10, where cardiac infiltrate is not evident yet, but immune responses are being initiated as shown by the rise in cardiac myosin-specific antibodies. Following day 10, CD45⁺ inflammatory cells begin infiltrating the cardiac space and peaks at day 21. This stage of disease mimics that of the secondary phase of CVB-induced myocarditis and is accompanied by anti-cardiac myosin antibodies and T-cells. Infiltration begins to resolve by day 35. The animal develops DCMI by day 45 as the inflammation is replaced by the deposition of collagen and other fibrotic mediators likely due to wound healing processes (Figure 2). EAM allows for the separation of the subsequent antigen-specific adaptive immune response independent of an active pathogen.

Figures and Tables

Table 1. Common Myocarditis Subtypes and Characteristics^{4,6,96}

Subtype	Myocyte Necrosis?	Diagnostic infiltrate	Secondary infiltrate
Lymphocytic	Y	Lymphocytes	Plasma cells, neutrophils, macrophages
Giant cell	Y	Giant cells	Lymphocytes, eosinophils
Eosinophilic	Y	Eosinophils	Lymphocytes

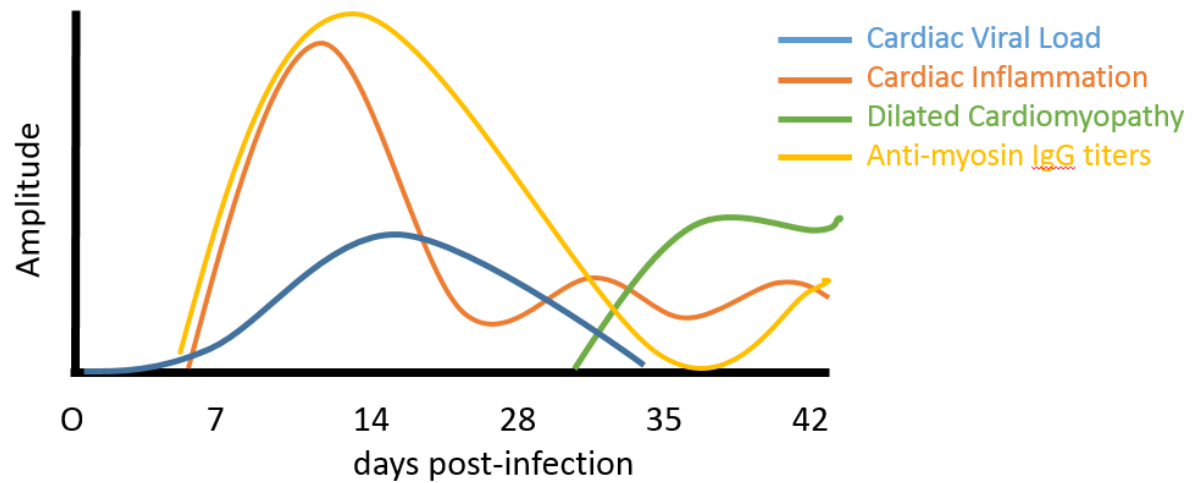
Table 2. Common etiologies of myocarditis^{4,6,37,39,96}

Infectious	Immune-mediated	Toxin-mediated
<u>Viral</u>	<u>Autoimmune</u>	Ethanol
Adenovirus	Celiac disease	Arsenic
Coxsackievirus	Churg-Strauss syndrome	Copper
Cytomegalovirus	Hypereosinophilic syndrome	Thyreotoxicosis
HIV	Inflammatory bowel disease	
Parvovirus	Kawasaki disease	
	Systemic lupus erythematosus	
<u>Bacterial</u>	<u>Hypersensitivity reactions</u>	
Streptococcus	Penicillin	
Mycobacterium	Tetracyclines	
Legionella	Benzodiazepines	
<u>Protozoan</u>		
Trypanosoma cruzi		
Toxoplasma gondi		

Table 3. Susceptible strains of mice and their associated peptides in experimental autoimmune myocarditis^{84,94}

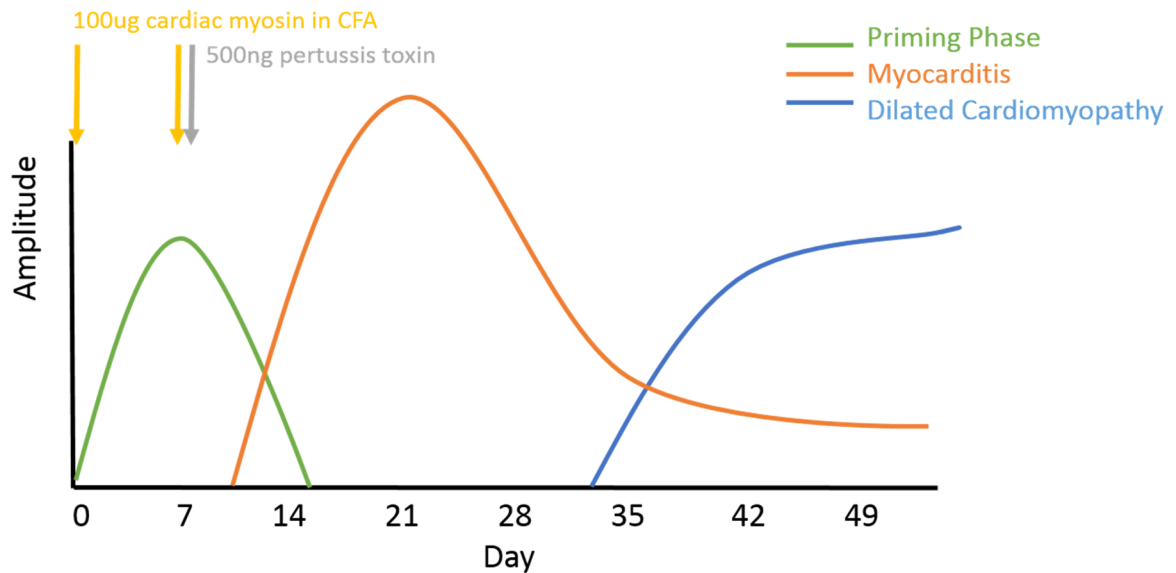
Antigen	Strain	1° Amino Acid Sequence
cardiac myosin	all	
MyHC $\alpha_{334-352}$	A/J, C3H (H2 ^k)	DSAFDVLSFTAEEKAGVYK
MyHC $\alpha_{614-629}$	BALB/c (H2 ^d)	Ac-SLKLMATLFSTVASAD
MyHC $\alpha_{406-425}$	SWXJ (H2 ^s)	KVGNEYVTKGQSVQQVYYSI
MyHC $\alpha_{1631-1650}$	SWXJ (H2 ^s)	LSQANRIASEAQKHLKNSQA
cardiac troponin I	A/J, BALB/c	
cTnI ₁₀₅₋₁₂₂	A/J (H2 ^k)	VDKVDEERYDVEAKVTKN

Figure 1. Natural History of Heart-Passaged CVB-Myocarditis^{85,86}



Heart-passaged coxsackievirus B (CVB) (Nancy strain) evokes myocarditis in BALB/c mice. Inoculated mice develop a disseminated viral infection for the first seven days until the virus invades cardiac tissue, where viral titer will peak around the same time as the ensuing cardiac inflammation. This coincides with anti-cardiac myosin IgG titers in the serum of infected mice. Inflammation will subside by day 28 of infection, where the mice develop a moderate chronic inflammation. The development of inflammatory dilated cardiomyopathy (DCMI) begins around day 35.

Figure 2. Progression of experimental autoimmune myocarditis^{84,94}



Experimental autoimmune myocarditis (EAM) is instigated when susceptible strains of mice are immunized with cardiac myosin alpha heavy chain protein or peptide emulsified in complete Freund's adjuvant (CFA) on days 0 and 7 of EAM. Mice undergo a priming phase from days 0-7, followed the beginnings of cardiac inflammation which peaks at day 21. Finally, DCMI occurs from day 42 onwards.

CHAPTER II: METHODS AND MATERIALS

Mice

WT BALB/c, Rag1^{-/-}, C.Cg-*Gata1*^{tm6Sho/J}, IFN γ ^{-/-}, IFN γ R1^{-/-}, and CCR3^{-/-} mice were purchased from the Jackson Laboratory and were bred and maintained in the conventional housing facilities at The Johns Hopkins University School of Medicine (Baltimore, Maryland). All protocols have been reviewed and approved by the Johns Hopkins Animal Care and Use Committee.

Immunization with MHC₆₁₄₋₆₂₉

Male 6-8 week old BALB/c mice were subcutaneously injected with 100 μ g of myocarditogenic peptide of cardiac myosin heavy chain (MyHC) MyHC₆₁₄₋₆₂₉, emulsified in an equal volume of complete Freund's adjuvant (CFA) (Sigma Aldrich, St. Louis, Missouri), supplemented with 4mg/ml of H37Ra tuberculosis extract (Difco, Lawrence, Kansas) on days 0 and 7 for a total volume of 100 μ L per injection⁹⁴. 500ng of Pertussis toxin in 100 μ L of PBS (Sigma Aldrich, St. Louis, Missouri) was administered intraperitoneally (i.p.) on day 0.

Assessment of EAM

Mice were sacrificed on day 14 or day 21 as required. Hearts were perfused with 1X phosphate buffered saline (PBS) for 3 minutes, removed and fixed. Longitudinal sections through the left ventricle were stained with hematoxylin and eosin (H&E)^{140,141,231,256}. The degree of myocardial infiltration and fibrosis was determined blindly by at least two individuals by microscopy. Histology scored based on percentage of infiltrated tissue as follows: 0 = no infiltration, 1 = \leq 10%, 2 = 10-30%, 3 = 30-50%, 4 = 50-90%, 5 \geq 90%⁹⁴.

Assessment of collagen deposition

Hearts at day 21 of EAM were perfused with 1X PBS as in for the assessment of EAM. Cardiac sections were stained with Masson Trichome^{140,141,231,256}. Images of whole cardiac sections were uploaded to ImageJ software (National Institutes of Health, Bethesda, Maryland) and using the loupe tool, the left ventricle was selected. Blue and red pixels within the selected area were enumerated and the degree of fibrosis represented as a percentage of blue pixels from the total number of pixels (red and blue).

Echocardiography

An Acuson Sequoia 256 high-resolution microimaging system with a 13 MHz transducer was used (Visualsonic, Toronto, Ontario, Canada). Any fur on the abdominal surface of the mice was removed using a depilatory cream, Nair (Church and Dwight, New Jersey, USA) 24 hours prior to imaging. In conscious mice, the heart was imaged in the 2D mode in the parasternal short-axis view. From the M-mode, the left ventricular wall thickness and chamber dimensions were measured. The M-mode cursor was positioned perpendicular to the interventricular septum (IVS) and the left ventricular posterior wall (LVPW) with three to five readings taken for each measurement. The left ventricular end diastolic dimension (LVEDd), end systolic dimension (LVEDs) and the interventricular septal wall thickness at end diastole (IVSD) were measured from a frozen M-mode tracing. Fractional shortening (FS), ejection fraction (EF) and relative wall thickness were calculated²⁵⁶.

Intracardiac and splenic flow cytometry

To create a cardiac single-cell suspension, the aorta was cannulated with a 23-gauge needle to perfuse hearts with 15mL of cold 1x PBS for 3 minutes to remove blood. To create cardiac single-cell suspensions, hearts were bisected, placed in C-tubes, and dissociated on the GentleMACS system (Miltenyi Biotech, Bergisch Gladbach, Germany) under program heart_01. Cells were shaken with 10mg of collagenase II and 1.5mg of DNase I (Worthington Biochemical, Lakewood, New Jersey) for 30 minutes at 37C. Cells were dissociated again under program heart_02 and rinsed twice with 1x PBS with 0.05% bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, Missouri) and 2mM ethylenediamine tetraacetic acid (EDTA) (Corning Cellgro, Corning, New York).

To create a splenic single-cell suspension, spleens were dissociated between two frosted glass slides and incubated with 2mL of ammonium-chloride-potassium (ACK) Lysing Buffer for 1 minute to remove red blood cells. The remaining cells were rinsed with 1x PBS and filtered through a 40uM mesh. $1-3 \times 10^6$ cells were incubated with 1uL of LIVE/DEAD Aqua (Invitrogen, Carlsbad, California) for 30 minutes in 1x PBS to stain dead cells. Cells were then incubated with 2 μ g of α CD16/32 at 4°C for 10 minutes prior to the addition of fluorescent antibodies (CD3, CD4, CD8, CD45, Ly6G, SiglecF, NKp46, DX5, CD11b, CD11c, and F4/80) (eBioscience, San Diego, California) to prevent non-specific binding of fluorescent antibodies. Samples were incubated with fluorescent antibodies at 4°C for 10-20 minutes, washed in 1ml of 0.05% BSA and 2mM EDTA in 1x PBS and fixed in fixation and permeabilization buffer (BD Bioscience, Franklin Lakes, New Jersey) for 30 minutes.

For intracellular cytokine staining, suspensions were first incubated prior to staining for

4-6 hours with 20ng/mL PMA, 1ug/mL ionomycin, and Golgistop (BD Bioscience, Franklin Lakes, New Jersey) in complete Dulbecco's Modified Eagle's Medium (DMEM) (Corning Cellgro, Corning, New York). Cells were surface stained and then permeabilized with 1x permeabilization buffer (BD Bioscience, Franklin Lakes, New Jersey) overnight at 4°C. Cells were then incubated with anti-cytokine antibodies (eBioscience, San Diego, California) for 30 minutes at 4°C. Cells were washed in 1x permeabilization buffer and resuspended in 100-200µl of buffer.

Samples were acquired on the LSR II quad-laser cytometer running FACSDiva 6 (BD Immunocytometry, Franklin Lakes, New Jersey). Data were analyzed with FlowJo 7.6 (Treestar Software, Ashland, Oregon).

Depletion of NK cells with anti-asialo GM1 antibody

To deplete NK cells prior to immunization, 6 week old male BALB/c mice were injected intraperitoneally (i.p.) with 1mg of anti-asialo GM1 in 200uL of sterile 1x PBS (Wako Chemicals USA, Richmond, Virginia) antibody everyday six days prior to the first immunization (days -6 to day 0)^{128,257}. To maintain decreased levels of NK cells after the first immunization (day 0), 1mg of anti-asialo GM1 antibody was administered every other day from day 1 until day 21 or sacrifice. Control mice received 1mg of rabbit IgG in 200uL of sterile PBS or 200uL of PBS alone (Sigma Aldrich, Saint Louis, Missouri) by the same schedule.

Isolation of primary adult mouse cardiac fibroblasts

Hearts dissected from 6-8 week old naïve BALB/c mice were perfused through the aorta

with warmed 37°C calcium-free buffer followed by collagenase type II (Worthington Biochemical, Lakewood, New Jersey) for 15 minutes. Tissue was dissolved into a single cell suspension and filtered through a 70µM mesh. Cells were seeded onto polystyrene plates at 37°C and non-adherent cells washed off after 1 hour. Cells were either collected immediately in Trizol reagent (Invitrogen, Carlsbad, California) for *ex vivo* experiments or passaged twice before *in vitro* use in complete DMEM with 20% FBS (Hyclone Laboratories, Logan, Utah), 1x penicillin/streptomycin, 25mM HEPES, 1x Anti-Anti, and 1x non-essential amino acids. In killing experiments, fibroblasts were treated with 1µM of angiotensin II in complete DMEM for 1 hour. Cells were then washed prior to the next step of experiment.

Isolation of primary NK cells

NK cells were negatively isolated from naïve or polyI:C-treated (1mg for 24 hours) WT or Rag1^{-/-} BALB/c spleens by manual magnetic cell sorting using the Mouse NK Isolation Kit II (Miltenyi Biotech, Bergisch Gladbach, Germany) and cultured for 24 hours with IL12 (10ng/mL) and IL15 (5ng/mL).

mRNA

For qPCR, cells or tissues were homogenized in TRIzol reagent (Invitrogen, Carlsbad, California) and chloroform extracted. Samples were DNase treated and cDNA libraries made using iScript Reverse Transcriptase Supermix (BioRad, Hercules, California). mRNA was amplified using Sybrgreen (Applied Biosystems, Foster City, California) and all values were calculated against HPRT mRNA. Values were controlled against isotype

control groups and shown as a function of fold induction using the formula $2^{-\Delta(\Delta CT)}$.

ELISA

Samples were run using tissue homogenates in 1x PBS or cell culture supernatant using enzyme-linked immunosorbent assays (ELISA) kits for CCL11 (RND Biosystems, Minneapolis, Minnesota) or a multiplex LINCO kit (Millipore, Jaffery, New Hampshire).

Statistics

Multiple group comparisons were performed by ordinary one-way ANOVA, followed by the Tukey-Kramer post-test (if parametric), or Kruskal-Wallis, followed by Dunn's post-test (if non-parametric) (Graphpad Prism, San Diego, California). All statistics of 2 groups were performed by student's t-test. P-values less than 0.050 were considered statistically significant.

CHAPTER III: NK CELLS ARE PROTECTIVE IN EAM

INTRODUCTION TO NK CELLS IN MYOCARDITIS

Biology of Natural Killer Cells

Natural killer (NK) cells comprise part of the Type 1 compartment of the innate lymphoid cell (ILC) family^{4,97}. Other ILCs include Th2-associated Type 2 ILCs and the Rorγt⁺ ILCs. ILCs are morphologically lymphocytic in nature, but lack the somatic rearrangement of antigen receptors found in the more classical T-cells and B-cells of the adaptive immune system. ILCs are responsive to multiple immune signals, are required for defense against pathogens, and are necessary for the formation of lymphoid organs, for repair to damaged tissue, and in tissue homeostasis⁹⁶.

NK cells contain pre-formed cytotoxic granules that are released upon specific activation signals that do not require previous sensitization and were originally classified as innate cytotoxic effector lymphocytes due to their stochastically expressed activation and inhibitory receptors and lack of memory formation^{98,99}. Since then, they have been recognized for their ability to modulate the immune system well-beyond the innate response, but they are still the first line of defense against many intracellular pathogens⁹⁹. The absence of NK cells results in increased viral titer and dissemination in multiple animal models^{16,23}.

NK cells classically recognize virally infected or tumor cells by their abnormal presentation of MHCI. MHCI on healthy cells results in the ligation of an inhibition receptor on the surface of NK cells, preventing the release of perforin and granzyme. However, NK cells have hundreds of invariant receptors that are stochastically expressed on each cell's surface during development that recognize a wide variety of self and foreign antigens^{25,27}. Certain viral proteins, such as influenza hemagglutinin (HA) and

Sendai HA-neuraminidase (HN), directly ligate NK activation receptors NKp46 and NKp44, respectively²³.

Mouse and human NK cells are believed to have arisen through convergent evolution¹². While many of the receptors expressed on murine and human NK cells recognize the same antigens and ligands, they have minimal sequence homologies^{100,101}. Therefore, functional comparisons between mouse and human NK receptors are difficult, though better comparisons can be made in terms of cytokine release and cytotoxic ability^{12,96}.

Development of Murine NK cells

NK cells and CD8+ T cells share similar development characteristics²⁶. Both arise from the bone-marrow sourced common lymphoid progenitor (CLP) and require signaling through the common- γ chain for survival and homeostasis²⁶. Unlike CD8+ T cells, however, NK cells do not require trafficking to the thymus for survival and activation. And while most NK cells arise and mature in the bone marrow, certain rare subsets of NK cells have been shown to finish maturing in the liver^{32,34}.

Unlike the established development of T-cells in the acquisition and loss of CD4 and CD8 markers during positive and negative selection in the thymus, NK development is characterized by the acquisition of arbitrary surface markers and functional ability as there has been no consensus reached yet on what the distinct maturation stages are (Figure 3). The earliest NK progenitors are non-stromal bone marrow cells known as pre-NK progenitors (pre-NKP) that express surface makers CD117 and CD244, and the

transcription factor Id2^{26,34,96,102}.

From this stage, the cells acquire CD122, the β -receptor subunit of both IL2 and IL15, and CD132, the common- γ chain. IL15 is required for the development and maturation of fully functional NK cells^{35,38}. At this time, it is unknown what signals control the shift from pre-NKPs to these NKPs, though transcription factors EOMES and T-bet are required¹⁰³. NKPs are defined as cells that express CD122 and CD132, but lack the functional capacities of mature NK cells and do not express other lineage markers such as CD3, CD19, and CD14. These cells are considered NK-committed and do not develop into other cell types upon *in vitro* stimulation^{52,104}.

NKP cells undergo the maturation process into immature NK cells (iNK) upon stimulation with IL15, a process that can be amplified with the addition of other CD122 and CD132 ligands IL3, IL7, c-kit ligand (KL), and flt-3 ligand (FL). The latter cytokines are not required for NK maturation, but are actively expressed by bone marrow stromal cells^{62,103}. iNK cells express high levels of CXCR4, a receptor for the highly expressed stromal-factor 1 in the bone marrow, and thus are retained until maturation⁶⁵. They also begin to express other NK-associated markers such as NKp46, NK1.1 (in C57B/6 mice), CD94, and CD27²⁶.

The exact mechanisms of NK maturation have not yet been well-delineated, though licensing of these cells through the ligation of MHCI with NK inhibitory receptors such as the Ly49 and KIR families is required¹⁰⁵. Without MHCI contact, immature NK cells remain anergic and nonfunctional, explaining why MHCI-deficient mice do not undergo massive inflammation yet have normal numbers of NK cells^{105,106}.

Mature NK cells acquire the expression of DX5 and CD11b, along with various

organ-specific chemokine receptors that may help egress from the bone marrow to specific sites in the body. These mature NK cells present with fluid organ-specific phenotypes through differential expression of chemokine receptors, cytokine secretions, and inherent cytotoxic abilities^{107,108}. Transfer of NK cells into another organ results in the conversion into the local NK phenotype¹⁰⁹.

Mature NK cells also acquire their canonical cytotoxicity functions, with preformed vesicles containing granzyme-B and perforin and can express cytotoxicity mediators Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL). Depending on the activation signals received, mature NK cells may also secrete various cytokines and inflammatory mediators, the most well-known by type I interferons, tumor necrosis factor alpha (TNF α), IFN γ , and IL13.

Involvement of Natural Killer Cells in Autoimmunity

NK cells have been found to accumulate in specific inflammatory sites in autoimmune diseases – in the joints of rheumatoid arthritis (RA) patients, in the skin lesions of psoriasis, and in the brain lesions of multiple sclerosis^{110,111}. Recently diagnosed type I diabetes patients were found to have NK cells localized around their pancreatic islets, though in post-mortem studies these pancreatic-infiltrating NK cells were not found. Similarly, RA patient joints contained higher proportions of activated CD56^{bright} and IFN γ production compared to periphery NK cells in the same individuals^{111,112}. When isolated and co-cultured with patient monocytes, these activated NK cells were able to induce differentiation of these monocytes into dendritic cells,

signifying an active role in the immune environment¹¹³. Furthermore, individuals with NK-lymphoproliferative disease of granular lymphocytes, a genetic condition resulting in the chronic accumulation of circulating NK cells, have increased risk of developing autoimmune manifestations^{114,115}.

This data contradicts with the observation that patients with RA, Sjogren's syndrome, and SLE have decreased circulating NK cell numbers and cytotoxicity potential^{112,116,117}. NK cells isolated from the periphery of RA patients failed to induce apoptosis in MHCI- deficient K562 cells compared to NK cells from the periphery of healthy controls *in vitro*. Additionally, MS patients in remission had higher proportions of activated NK cells in their periphery, as defined by increased Fas expression and Th2 cytokine secretions, than patients with active disease¹¹⁸. Altogether, it is not clear whether autoimmune diseases are exacerbated by the deficiency or excesses of NK cells, making animal studies necessary.

However, studies in animal models have also resulted in a plethora of conflicting data. Depending on the strain of mouse and method of NK manipulation, widely different disease outcomes can be observed. This is perhaps most evident in the best known of autoimmune disease models – experimental autoimmune encephalomyelitis (EAE).

EAE is a model of multiple sclerosis and can be induced by immunization with myelin oligodendrocyte-glycoprotein (MOG), myelin basic protein (MBP), etc¹¹⁹. Depletion of NK cells from C57B/6 mice in whole protein MOG-induced EAE by NK1.1 antibodies resulted in aggravation of disease and increased Th1 infiltration into the CNS. Furthermore, transfer of NK cells into mice undergoing EAE reduced disease severity¹²⁰. However, other studies in IL18 deficient mice noted that IFN γ -producing NK cells were

required for the progression of disease and that depletion of NK cells from MOG-peptide induced EAE using NK1.1 or anti-asialo GM1 depressed disease^{117,121}.

Similar contradictions have been found in autoimmune animal models of insulin-independent diabetes-mellitus, colitis, and collagen-induced arthritis^{110,111,113}. Diabetes can be prevented in non-obese diabetic (NOD) mice by subcutaneous administration of CFA, which induced NK trafficking and secretion of IFN γ . Depletion of NK cells rendered CFA administration inconsequential in protection against disease development¹²². However, in two other models of diabetes, BDC2.5/NOD and BDC2.5/B6^{g7}, associations were found between NK cell numbers in the pancreas and levels of insulinitis, and depletion of NK cells limited disease severity^{123,124}.

In studies of NK cells both clinically and in animal models, some contradictory results could be attributed to difficulties in separating the NK and natural killer T-cell (NKT) populations. NKT cells share many of the same functions and mechanisms of NK cells, with the addition of a somatically rearranged T-cell receptor (TCR)^{125,126}. NK1.1 antibody, a widely used method for NK depletion in C57Bl/6 mice, also depletes NKT populations¹²⁶. Anti-asialo GM-1, on the other hand, used in BALB/c mice models, leaves the NKT component untouched^{127,128}.

NK cells are distinct controllers of disease and dysregulation in their biology can dramatically shift clinical outcomes. Furthermore, the mechanisms by which NK cells alter disease are not limited to cytotoxicity. Cytokine release resulting in alteration of Th subtypes, direct contact-mediated lysis of auto-aggressive T cells, and accelerated maturation of monocytes and dendritic cells are all ways by which NK cells modulate the innate and adaptive immune system. Therefore, NK cells may in either protective or

pathogenic in progression of autoimmune disease. The lack of a consensus phenotype across autoimmune features may indicate that the role of NK cells may largely hinge on the target organ involved, as NK cells are phenotypically very diverse and are greatly influenced by their local microenvironment.

Role of Natural Killer Cells in Myocarditis

Similar to other autoimmune clinical studies, both idiopathic and viral myocarditis patients have severely decreased levels of natural killer (NK) cells and non-MHC restricted toxicity, pointing towards defects in both frequency and function^{129,130}. The same was also found in DCM patients, though whether these DCM patients were secondary as a result of primary myocarditis is not stated¹³¹. Perforin-caused pores on the surface of virally-infected myocytes have been found in both clinical myocarditis patients and in animal viral myocarditis models¹³². In contradiction, heart biopsies of 18 early myocarditis patients did not reveal any NK cell infiltration, though it is unclear whether this is due to the sampling limitations¹³³

NK cells play an essential role in the defense against acute viral pathogens associated with clinical myocarditis. We and another groups have reported NK cells as protective against both CV3 and MCMV-induced myocarditis, though it must be said that these studies depleted both NK and NKT populations simultaneously^{85,134}. The direct mediators of viral recognition are unknown in CBV and MCMV, though ligation of activating receptor Ly49H by the viral glycoprotein m157 and destabilization of inhibitory receptor Ly49C by abnormal MHCI expression is necessary in limiting

MCMV infection^{135,136}.

NK1.1 antibody treatment in normally chronic myocarditis-resistant strains C57BL/6 and BALB.B6-*Cmv*^{1r} mice, which express NK1.1 on their NK cells, resulted in disease progression mimicking the natural progression of susceptible BALB/c MCMV- and CBV-induced viral myocarditis⁸⁵. This indicates that NK cells play a large role in the determination of strain-dependent resistance to experimental myocarditis. It is unknown if NK cells act solely by limiting viral replication in myocarditis, reducing the autoimmune response, or both.^{137,138}

If NK cells control both viral load and inflammation in myocarditis, they would be an excellent candidate for targeted biologic therapy. Patients would no longer have to be stratified by the presence of virus in the heart and autoantibodies in the serum in order to determine their course of treatment as NK cells would be able to target both mechanisms, viral and autoimmune, simultaneously. In essence, NK cells or NK-derived products concurrently could serve as an anti-viral and an immunosuppressant.

RATIONALE

We undertook the research in this dissertation in order to determine if natural killer (NK) cells or their products could modulate the pathogenesis of myocarditis in the absence of virus. While the protective role of NK cells in the advent of CBV or MCMV-induced myocarditis was largely attributed to reduction of viral titer and prevention of inflammatory cascades, it is possible the NK cells may have limited the inflammation in a virus-independent manner^{85,93,139}. EAM is a largely Th-driven disease and NK cells have been shown to affect polarization of Th subsets through secretion of IFN γ and by killing of activated antigen-specific T cells. Both of those actions would be protective in EAM, as IFN γ -/- mice had significantly worse disease and that CD4 myosin-specific T cells are the driver of inflammation^{94,140,141}. Therefore, we hypothesized the NK cells would be protective in EAM. Furthermore, given the ability of NK cells to control non-pathogenic models of liver fibrosis through the killing of activated fibroblasts, we expected that NK cells would limit collagen deposition in the heart^{121,123,124}.

RESULTS

NK cells accumulate in the heart during EAM

To examine the kinetics of NK cell accumulation during cardiac inflammation, we induced EAM in male wild type (WT) BALB/c mice. Levels of NK cells in perfused hearts at day 21 of EAM by flow cytometry on days 0, 14, and 21 were assessed (Figure 4A). The numbers of CD5⁻DX5⁺NKp46⁺ NK cells significantly increased from day 0 to days 14 and 21 during EAM in absolute counts, however the proportion of NK cells out of CD45⁺ cells in the heart was stable (Figure 4B and 4C). Thus, NK cell comprised a distinct population in the heart that actively accumulated during EAM.

Cardiac NK cells secrete IFN γ and cytotoxic mediators

To determine NK cell phenotypes in the heart, we profiled cytokine and cytotoxicity mediators NK cells by flow cytometry at the peak of inflammation on day 21 of EAM and compared them against periphery NK cells from the spleen. There were 6-fold more cardiac NK cells secreting IFN γ compared to splenic NK cells (Figure 5A). The two populations had equivalent IL13 expression (Figure 5B) and neither expressed IL5 (*data not shown*). Compared to spleen NK cells, significantly greater proportions of cardiac NK cells expressed perforin and were positive for LAMP-1, a correlate for granzyme B secretion (Figure 5C and 5D)¹⁴². Cardiac NK cells had increased IFN γ and cytotoxicity potential when compared against peripheral NK cells at the same time.

Cardiac NK cells upregulate certain activation markers

We also assessed the expression of various activation and inhibitory surface markers on cardiac NK cells compared to peripheral splenic NK cells to determine if they had a distinct phenotype. Cardiac NK cells had upregulated levels of the activation markers CD27, TRAIL, and CD69 (Figure 6A, 6B, and 6C) with decreased levels of NKG2D (Figure 6D). Cardiac NK cells also upregulated levels of Ly49A/D and Ly49H (Figure 6E and 6F). Cardiac NK cells can be differentiated from peripheral NK cells by their surface marker profiles.

ASGM-1 treatment in naïve animals selectively depletes NK cells

To determine if NK cells could be depleted using anti-asialogangloside GM-1 (ASGM-1) polyclonal antibody treatment and how this treated affected other cellular populations in naïve BALB/c animals *in vivo*, mice were injected for 3 days with 1mg of ASGM-1 *i.p.* Mice were sacrificed at day 4 and lungs, hearts, livers, spleens, and blood were collected. Cell populations were measured by flow cytometry. ASGM-1 antibody treatment depleted CD3⁺DX5⁺NKp46⁺ NK, but did not affect CD3⁺DX5⁺NKp46⁺ NKT cells or Ly6G^{lo}SiglecF⁺ eosinophils (Figure 7A – 7C) in naïve animals.

ASGM-1 depletes NK, but not NKT cells during EAM

To determine whether NK cells could be depleted using ASGM1 throughout EAM, NK cells were depleted *in vivo* using 1mg of polyclonal antibody injected *i.p.* from day -6 to day 21 of EAM as described in the methods (Figure 8A). The ASGM-1 antibody treatment led to significant reductions in the number and proportion of CD3⁺

DX5⁺NKp46⁺ NK cells (Figure 8B) in the spleen and heart during EAM. This treatment schedule did not alter the frequency of the CD3⁺DX5⁺ NKT cell population. Therefore, ASGM-1 treatment, unlike NK1.1 antibody treatment in C57BL/6 mice, is specific for NK cells.

NK cells limit cardiac inflammation during EAM

We explored whether NK cells played a role in the progression of EAM by depleting NK cells throughout disease and sacrificing the animals at day 21 in order to examine levels of cardiac inflammation. ASGM-1 treatment resulted in significantly more severe myocarditis on day 21 as assessed by histology (Figure 9A). This was mirrored by increased CD45⁺ infiltration in the heart as measured by flow cytometry (Figure 9B). The more severe disease in NK-depleted animals was also accompanied by increased levels of circulating anti-MyHC $\alpha_{614-629}$ -specific IgG antibodies (Figure 9C).

ASGM-1 treated animals have increased fibrosis at day 21 of EAM

We observed accelerated cardiac fibrosis on day 21 in NK-depleted animals using Masson's Trichrome staining (Figure 10A). In order to assess fibrosis quantitatively, we processed all images of stained heart slides through ImageJ to enumerate the percentage of collagen-positive (blue) pixels versus non-fibrotic (red) pixels as described in the methods section. NK-depleted animals had increased levels of collagen deposition (Figure 10A), indicating that NK cells may protect against fibrosis.

We also quantified the active production of collagen in NK-depleted mice at day 21 of EAM, as this was early in the disease process and most likely, cessation of collagen

production often seen in late stages of DCMI had not occurred. Indeed, we found that NK depletion lead to increased production of collagen as seen by mRNA levels of collagen 1 and collagen 3 in the heart (Figure 11A – 11C).

ASGM-1 treatment during EAM mildly decreases cardiac function

To determine whether this increased collagen deposition in the heart had any functional consequences, we performed echocardiography on isotype control and ASGM-1 treated animals at day 21 of disease. NK-depletion worsened heart function as shown by significantly decreased cardiac ejection fraction (Figure 12A), which was attributed to changes in LV end systolic, but not diastolic, diameter (Figure 12B and 12C). This change was echoed in levels of fractional shortening (Figure 12D). No changes were seen in LV intraventricular septal thickness (Figure 12E). The deposition of collagen may have limited cardiac function by preventing heart contractility.

Activated fibroblasts are not targeted for cytotoxic killing by NK cells *in vitro*

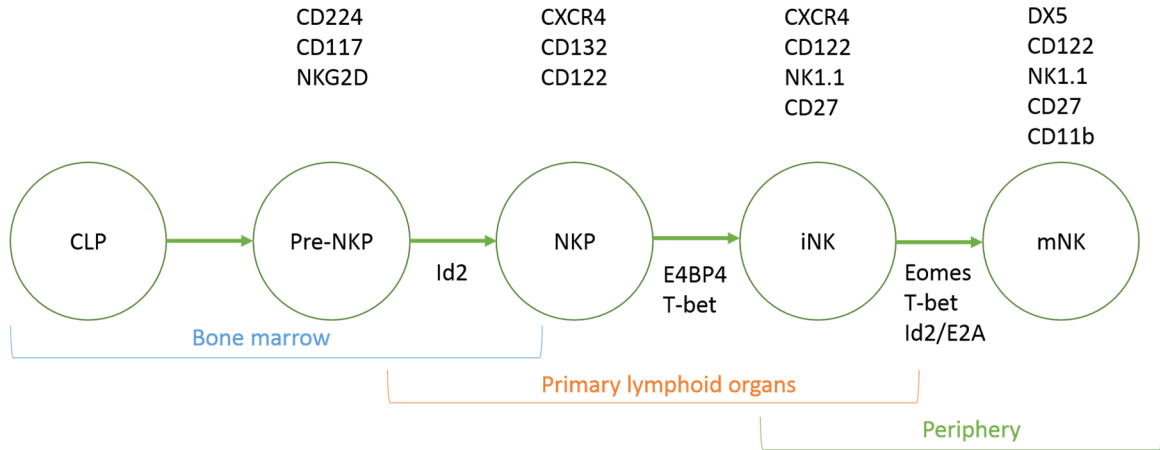
We explored whether NK cells mediated protection against cardiac fibrosis by killing activated resident fibroblasts as reported in models of liver fibrosis^{123,124}. We isolated adult mouse cardiac fibroblasts (CFs) from naïve BALB/c mice as described in the methods and passaged them twice to yield a pure population of CFs with positive staining for alpha smooth muscle actin (α -SMA), expression of fibroblast-associated markers fibroblast growth factor (FGF) and vimentin, and undetectable levels of myeloid or lymphocyte contamination as measured by CD11b and CD3 (Figure 13A – 13G).

Splenic NK cells were magnetically isolated in either naïve or activated states, the

latter being induced by polyI:C (1mg) treatment administered i.p. 24 hours prior as described in the methods. Activated NK cells had increased expression of NKG2D and CD69 compared to naïve cells (Figure 14A). CFs were first activated with angiotensin II (AngII) at 1uM for 1 hour, illustrated by increased vimentin mRNA (Figure 14B). CFs were then washed and NK cells were co-cultured with CFs for 48 hours in a 1:1 ratio. CFs were then stained green with calcein AM (live) and red with ethidium homodimer (dead) and examined using immunofluorescence microscopy. While NK cells induced cell death in CFs, no difference was seen between untreated and activated CFs (Figure 14C – 14E), indicating that NK-mediated killing of activated fibroblasts during EAM was not a likely mechanism of protection.

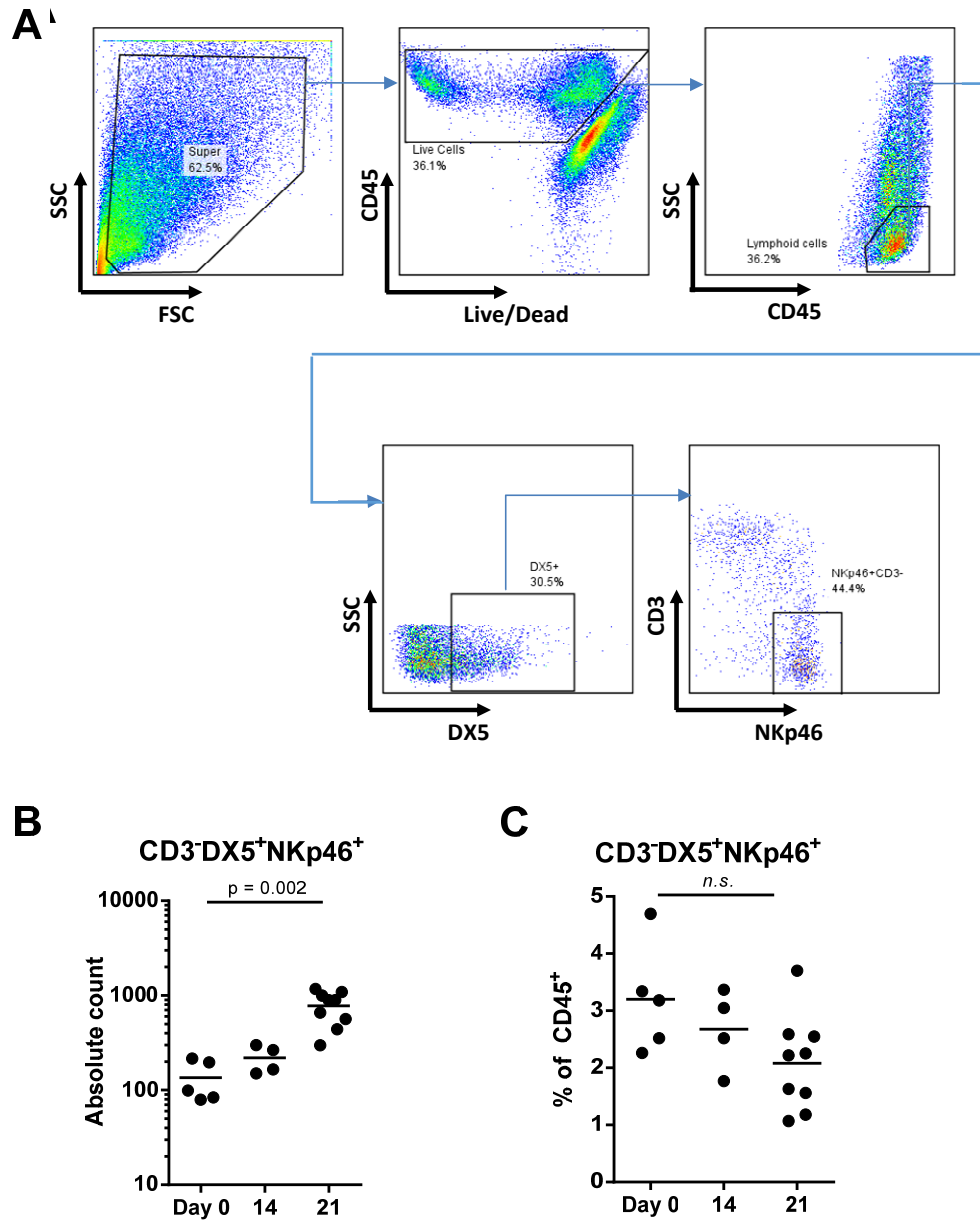
FIGURES AND TABLES

Figure 3. Maturation stages and associated transcription factors of murine NK cells



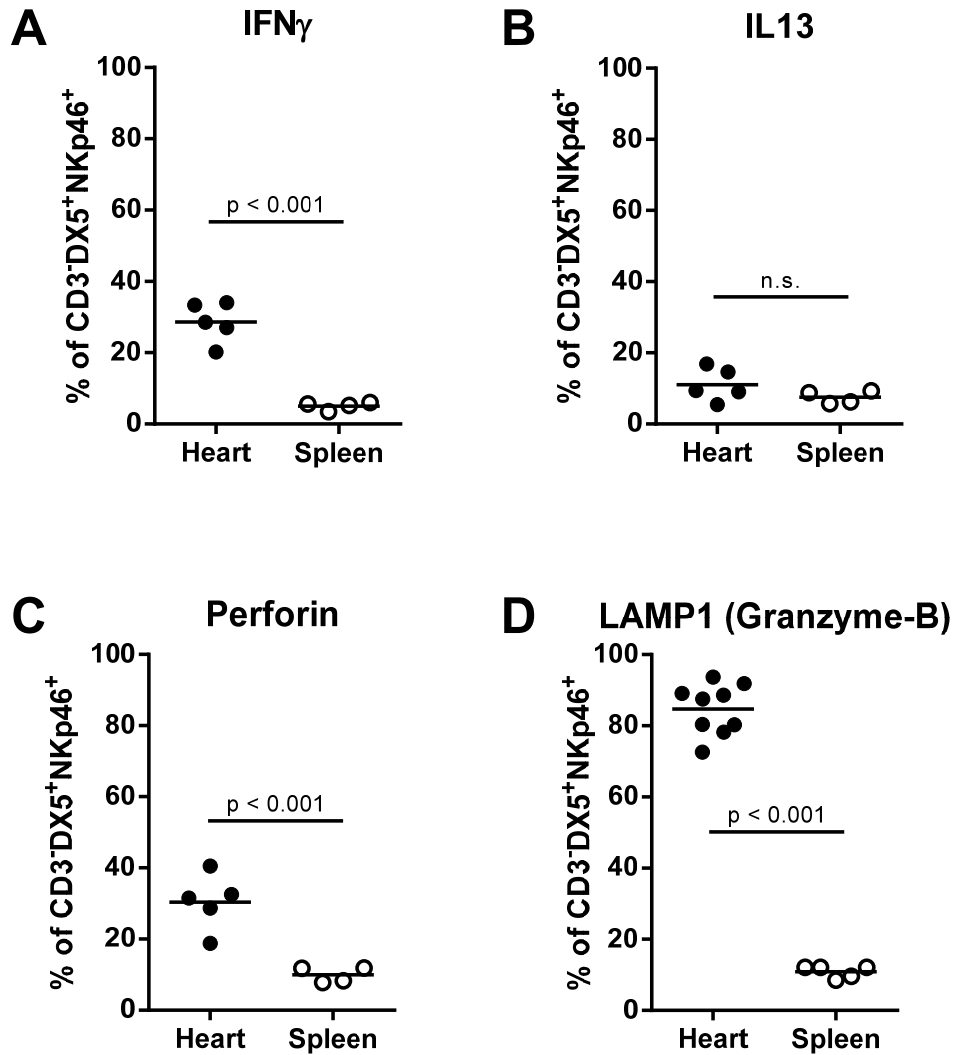
NK development is broken up to distinct stages based on surface marker expression, functional ability, and transcription factor expression. Common lymphoid progenitors in the bone marrow develop into NK-progenitors (NKPs) though transcription factors EOMES and T-bet are required. NKPs are committed and do not develop into other cell types. From this stage, NKP cells become immature NK cells (iNK) upon stimulation with IL15 under the control of T-bet and EOMES^{107,143,144}.

Figure 4. NK cells accumulate in the heart during EAM



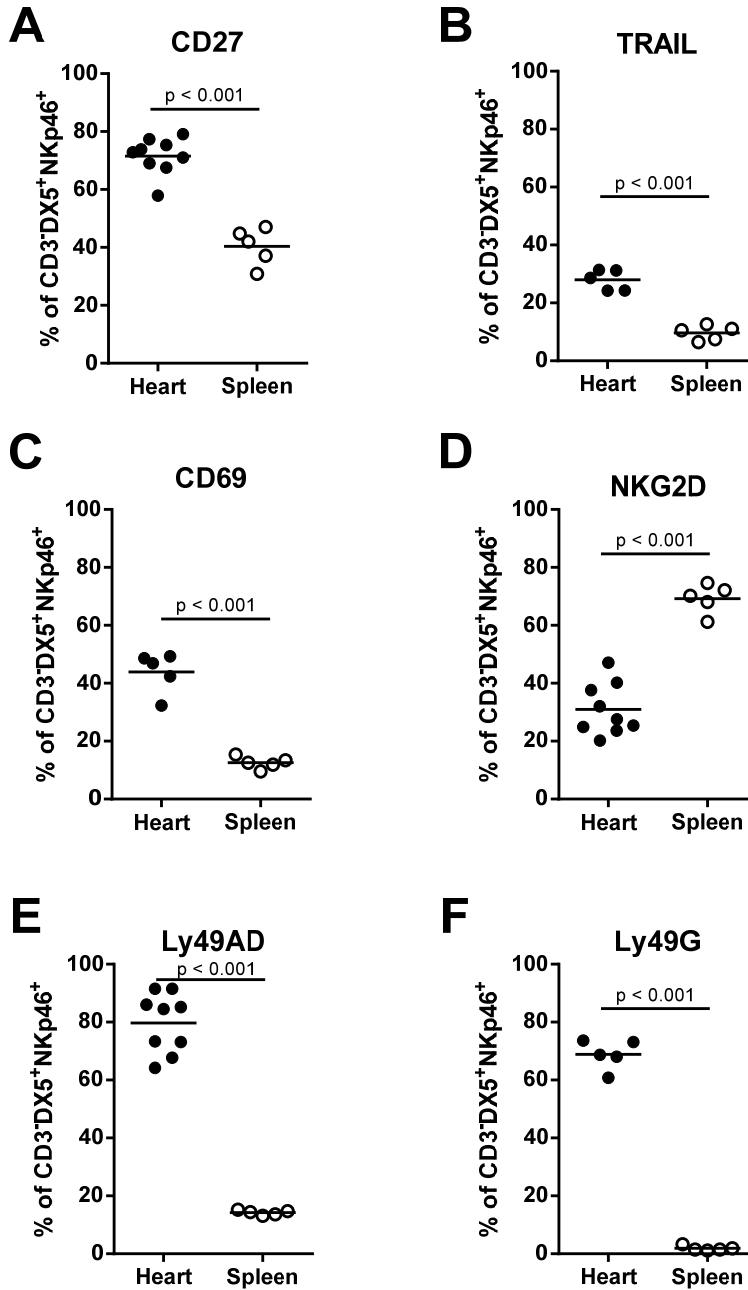
A) Representative gating strategy used for CD3-DX5+NKp46⁺ NK cells in the heart. B) Absolute numbers (ANOVA $p=0.002$) and C) percentages ($n.s.$) of CD3-DX5+NKp46⁺ NK cells infiltrating the heart during days 0, 14, and 21 of EAM by flow cytometry. Percentages based on total CD45⁺ cells in the heart. Significance by ordinary one-way ANOVA with post-testing by Tukey's multiple comparisons test.

Figure 5. Cardiac NK cells secrete IFN γ and cytotoxic mediators



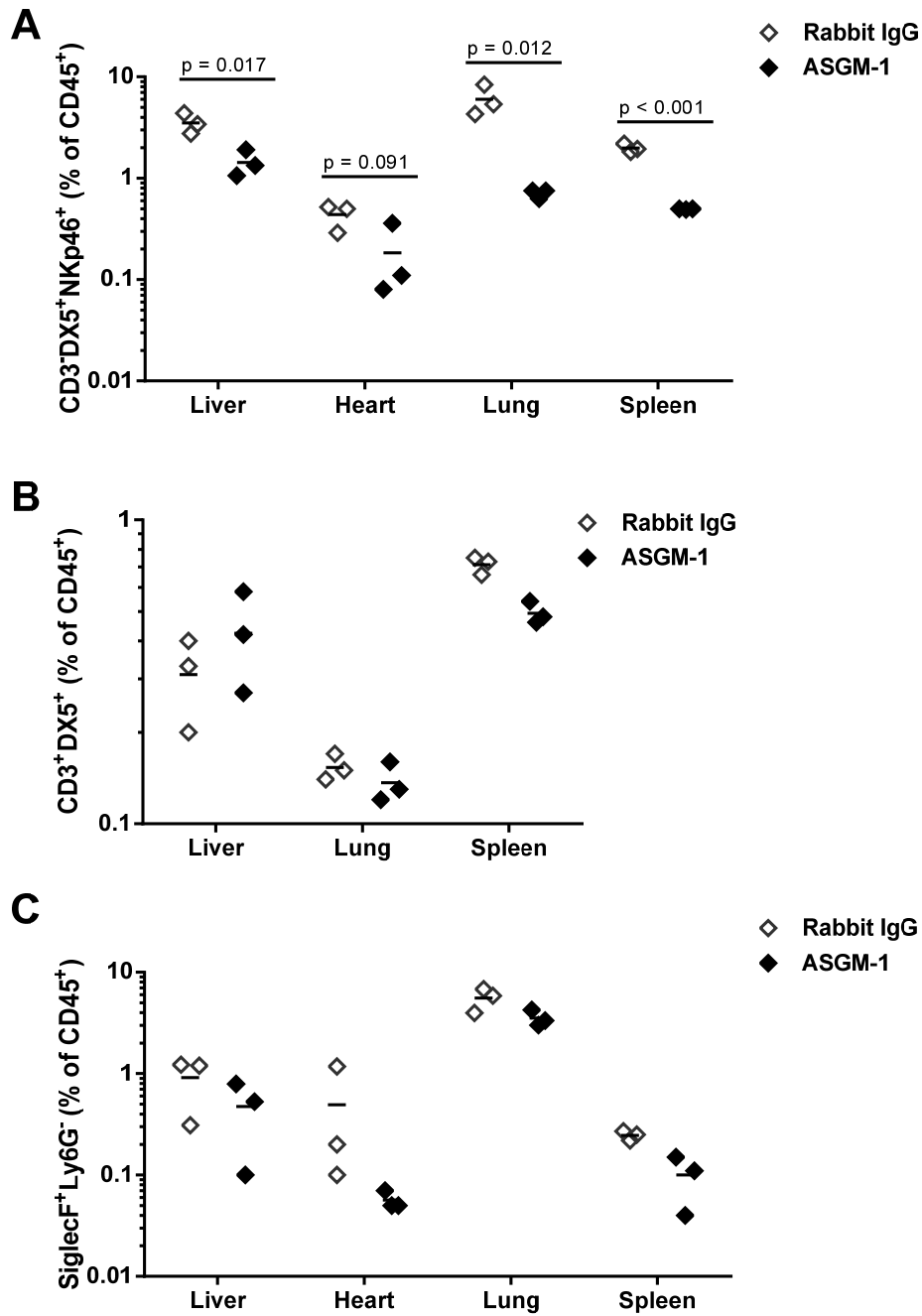
To determine NK physiology, cardiac and splenic CD3⁺DX5⁺NKp46⁺ NK cells were stained at day 21 of EAM. Percentage of A) IFN γ ($p < 0.001$), B) IL13 (n.s.), and C) perforin ($p < 0.001$) positive cells were based on positive intracellular antibody staining after 4-6 hours of PMA/ionomycin stimulation and Golgistop *in vitro*. D) Percentage of LAMP-1⁺, a marker for granzyme-B release, staining ($p < 0.001$).

Figure 6. Cardiac NK cells upregulate certain activation markers



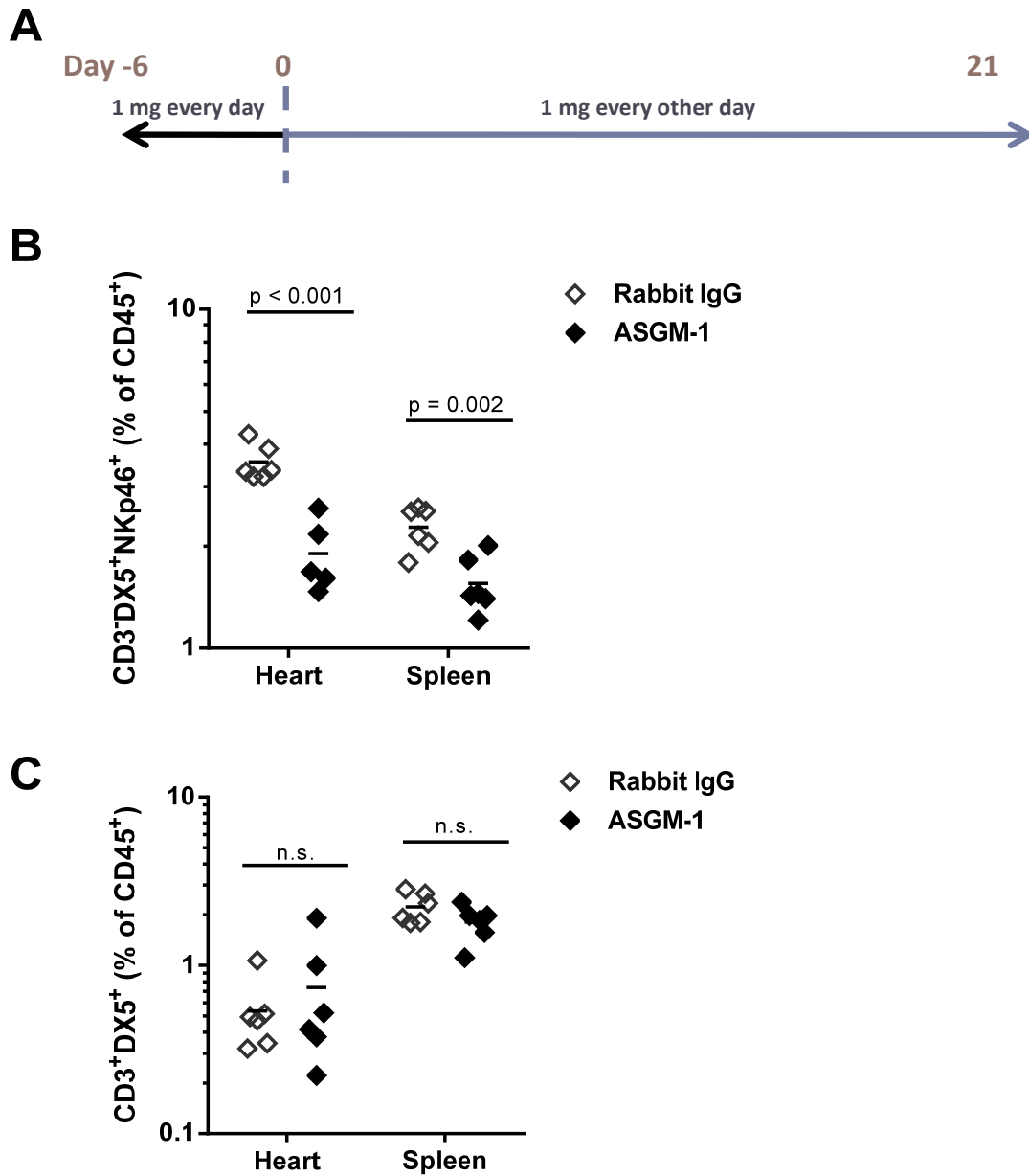
To determine the cell surface phenotype of cardiac and splenic NK cells at day 21 of EAM, NK cells were examined by flow cytometry. Percentage of NK cells positive for activation-associated markers A) CD27, B) TRAIL, C) CD69, and D) NKG2D. Percentage of NK cells positive for inhibitory receptors E) Ly49AD and F) Ly49G. Significance for A-F calculated by unpaired *t*-test.

Figure 7. ASGM-1 treatment in naïve animals selectively depletes NK cells



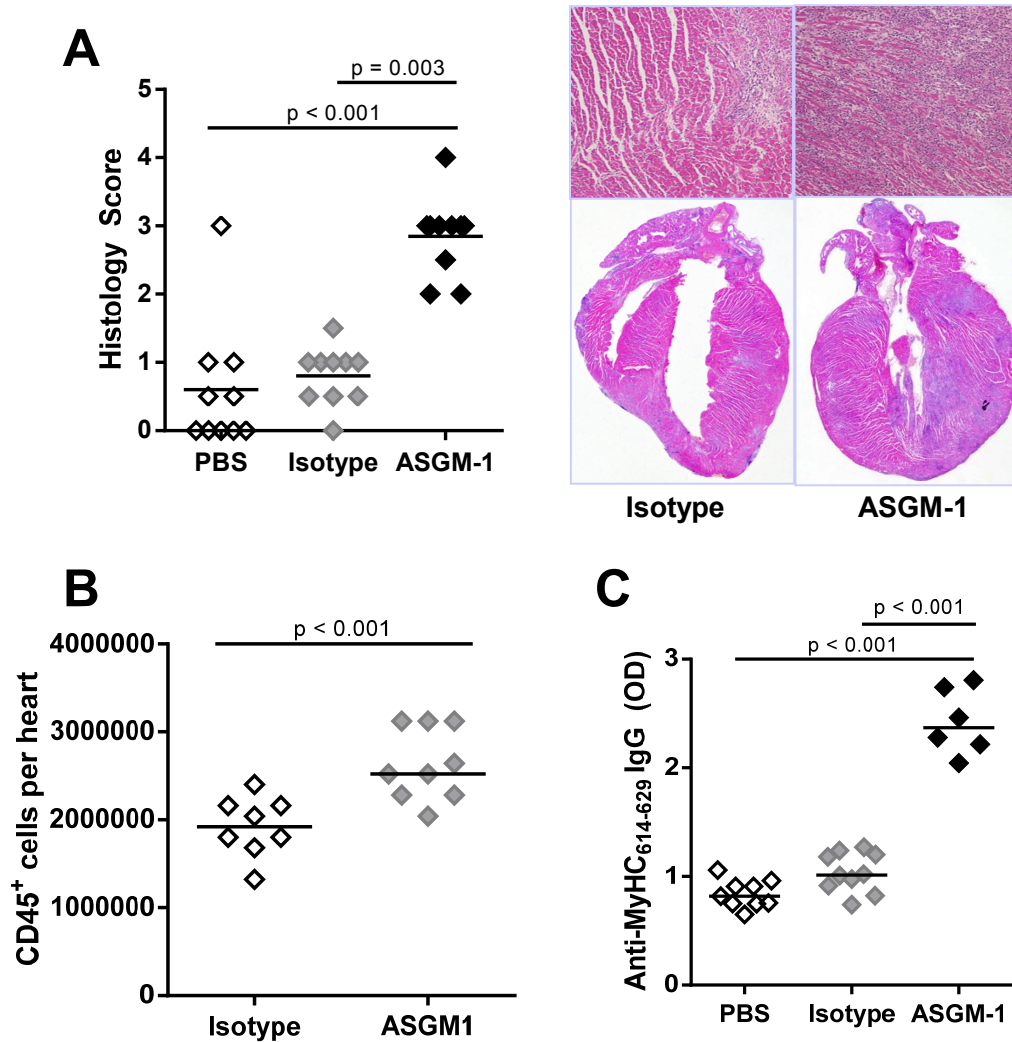
Percentage of A) CD3⁺DX5⁺NKp46⁺ NK cells, B) CD3⁺DX5⁺NKp46⁺ NKT cells (all *n.s.*), and C) Ly6G^{lo}SiglecF⁺ eosinophils (all *n.s.*) in the liver, heart, lung, and spleen of naïve WT mice after three days of rabbit IgG or ASGM-1 treatment of 1mg of antibody per day. All statistics calculated by unpaired t-tests.

Figure 8. ASGM-1 depletes NK, but not NKT cells during EAM



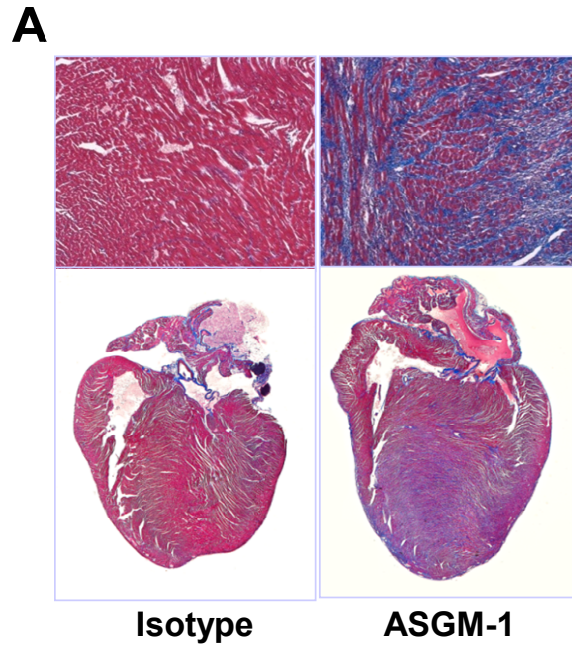
A) Schematic of rabbit IgG and ASGM-1 antibody treatment schedule throughout EAM. Percentage of B) CD3⁺DX5⁺NKp46⁺ NK cells in the heart ($p < 0.001$) and spleen ($p < 0.002$) and C) CD3⁺DX5⁺ NKT cells out of CD45⁺ cells (all n.s.) of rabbit IgG and ASGM-1 mAb treated WT mice at day 21 of EAM as assessed by flow cytometry

Figure 9. NK cells limit cardiac inflammation during EAM



A) Histology scores and representative histology of H&E stained cardiac sections from PBS, rabbit IgG, and ASGM-1 antibody treated animals at day 21 of EAM (ANOVA $p < 0.001$). B) Total CD45⁺ cells infiltrating the hearts of isotype control and ASGM-1 treated animals at day 21 of EAM as assessed by flow cytometry ($p < 0.001$). C) Levels of anti-MyHC₆₁₄₋₆₂₉ total IgG antibodies in the serum of PBS, isotype control, and ASGM-1 treated animals at day 21 of EAM (ANOVA $p < 0.001$).

Figure 10. ASGM-1 treated animals have increased fibrosis at day 21 of EAM



A) Representative histology of Masson's Trichrome stained cardiac sections from rabbit IgG, and ASGM-1 antibody treated animals at day 21 of EAM. B) Enumeration of fibrosis from Masson's Trichrome slides by calculation of red versus blue pixels on ImageJ software ($p=0.042$). Statistics calculated by unpaired t-test.

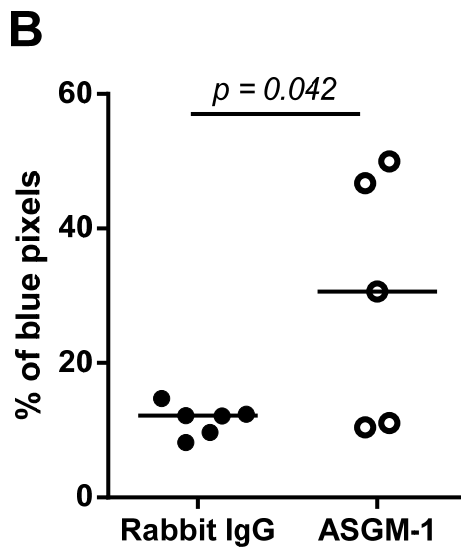
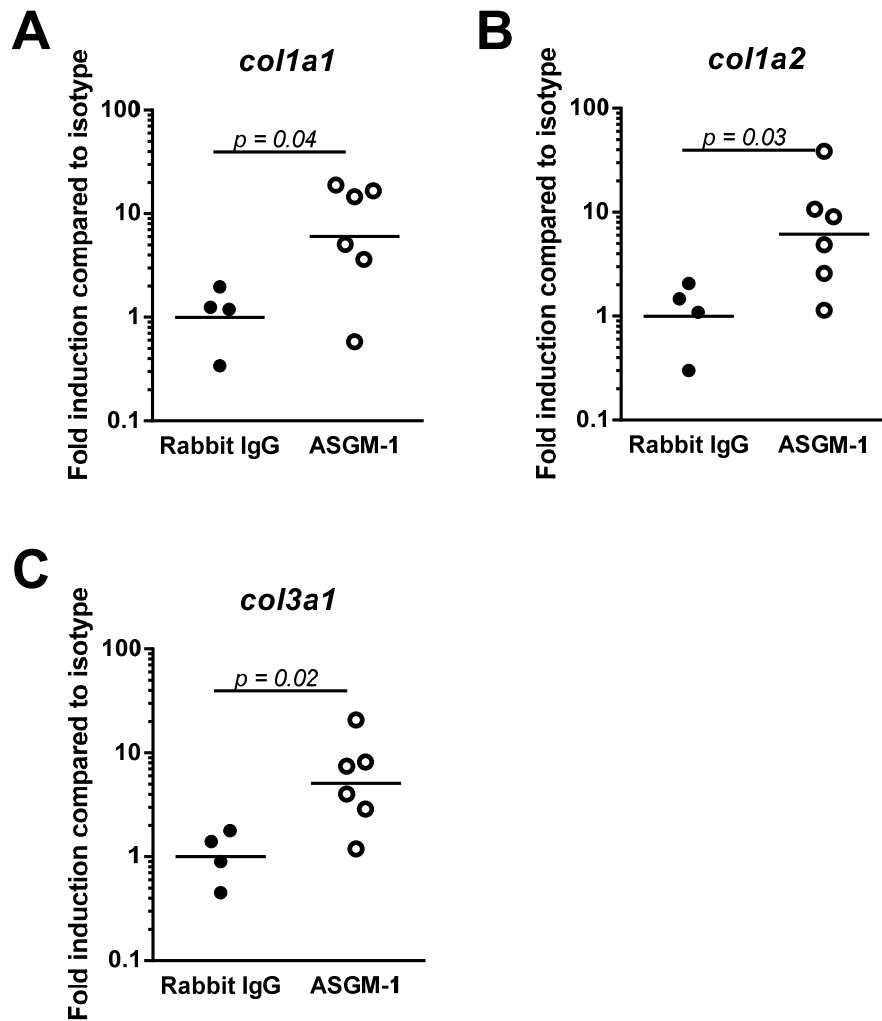
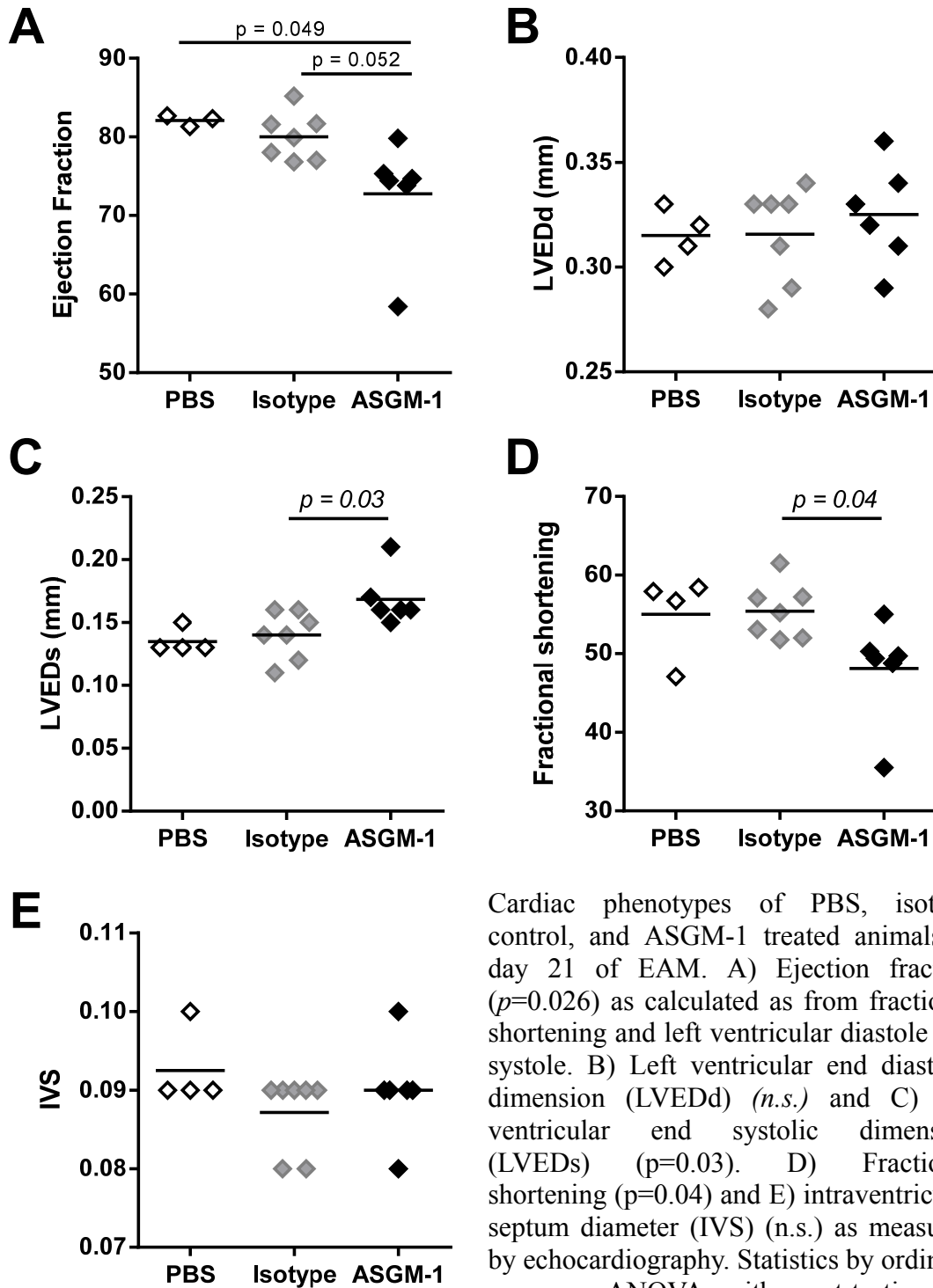


Figure 11. NK cells limit collagen deposition during EAM



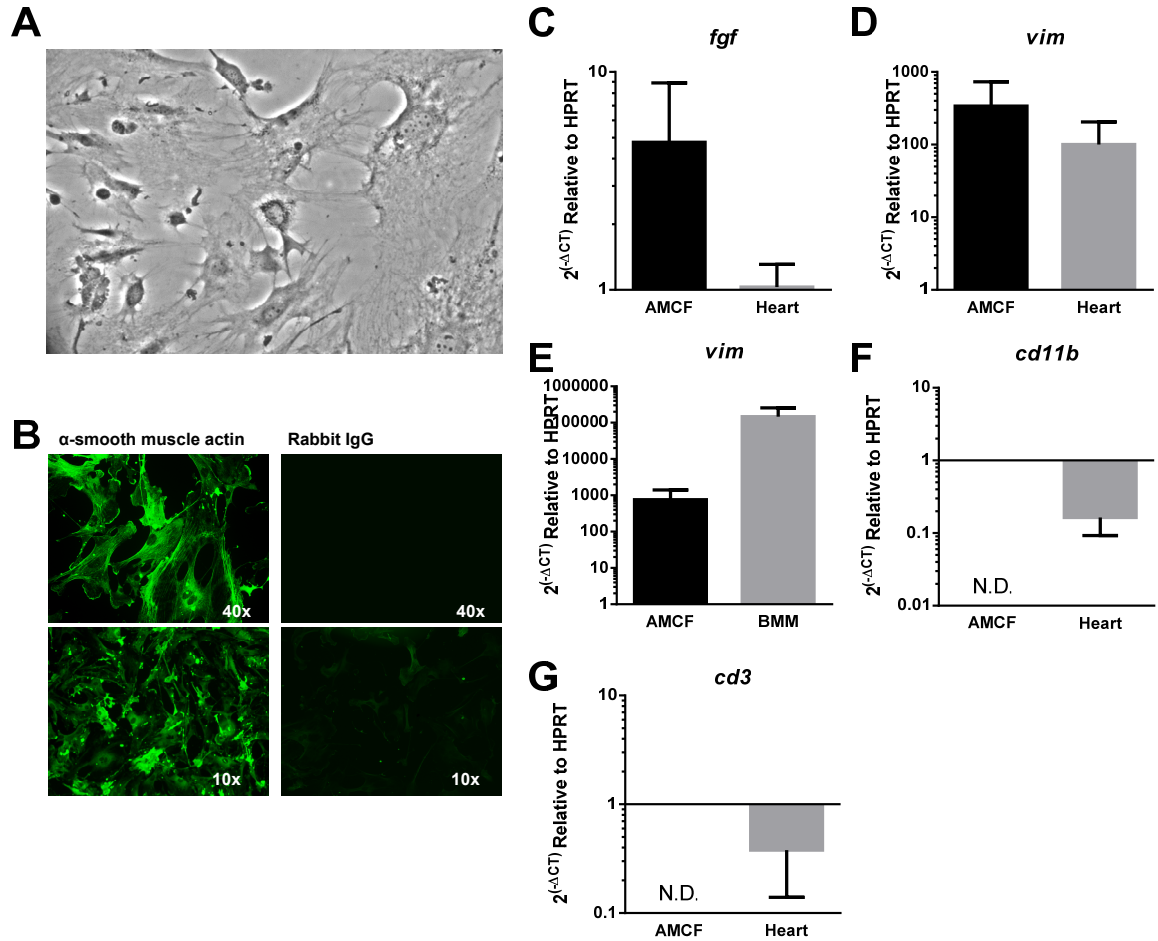
Collagen production as measured using qPCR for A) collagen 1a1 ($p=0.04$), B) collagen 1a2 ($p=0.03$), and C) collagen 3a1 ($p=0.02$) for mRNA in hearts of rabbit IgG and ASGM-1 treated animals at day 21 of EAM. Values calculated as a function of HPRT levels and compared against rabbit IgG. Statistics calculated by unpaired t-test.

Figure 12. ASGM-1 treatment during EAM mildly decreases cardiac function



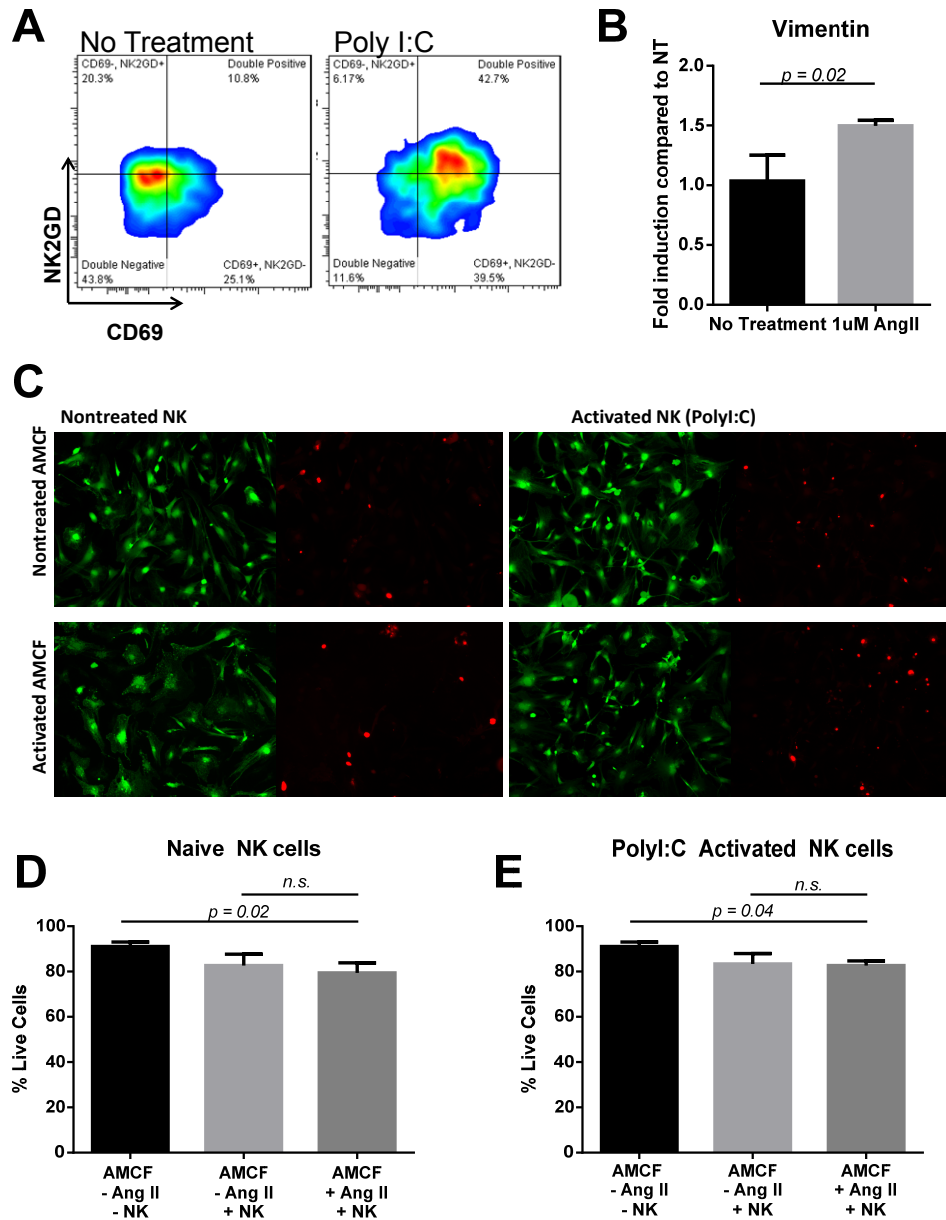
Cardiac phenotypes of PBS, isotype control, and ASGM-1 treated animals at day 21 of EAM. A) Ejection fraction ($p=0.026$) as calculated as from fractional shortening and left ventricular diastole and systole. B) Left ventricular end diastolic dimension (LVEDd) (*n.s.*) and C) left ventricular end systolic dimension (LVEDs) ($p=0.03$). D) Fractional shortening ($p=0.04$) and E) intraventricular septum diameter (IVS) (*n.s.*) as measured by echocardiography. Statistics by ordinary one-way ANOVA with post-testing by Tukey's multiple comparisons test.

Figure 13. Cardiac fibroblasts can be isolated from adult mice



A) Bright-field and B) immunofluorescence images of adult mouse cardiac fibroblasts (CFs) isolated and passaged twice, fixed with 4% paraformaldehyde and stained with 1:1000 anti-alpha smooth muscle primary rabbit antibody or rabbit IgG isotype antibody and 1:200 anti-rabbit rat FITC-conjugated antibody. Levels of C) fibroblast growth factor and D) vimentin mRNA as measured by qPCR relative to HPRT in untreated CFs and naïve BALB/c whole heart homogenates. Levels of D) vimentin, E) CD11b, and F) CD3 mRNA as measured by qPCR relative to HPRT in untreated CFs and bone marrow and M-CSF derived monocytes.

Figure 14. Activated fibroblasts are not targeted for cytotoxic killing by NK cells in vitro



A) Expression of NKG2D and CD69 in splenic NK cells isolated from naïve or polyI:C treated mice after 24 hours. B) Levels of vimentin mRNA measured by qPCR in isolated CFs after no treatment or angiotensin II (AngII) treatment (1uM) for 1 hour as controlled by HPRT ($p=0.02$). C) Representative images of live and dead no treatment and AngII treated CFs after 48 hours of 1:1 co-culture with naïve or polyI:C activated NK cells. Percentage of live CFs after 48 hours of co-culture with D) naïve and D) activated NK cells as determined by red versus green positive cells counted manually on ImageJ software. Statistics for B calculated by unpaired t-test and C and D by one way ANOVA with Tukey's post-testing.

DISCUSSION

Given the role of NK cells in the mediation of viral myocarditis and their newly understood ability to mediate the adaptive immune system, we hypothesized that NK cells may play a role in the pathogenesis of EAM. Indeed, we provide the first examination of inflammatory cardiac NK cells and show that they represent a distinct and activated population compared to NK cells found in the spleen. Their accumulation in the heart suggests that they are recruited during EAM, though the mechanism of recruitment is unknown. It is postulated that their recruitment and trafficking is dependent on the target cell whether tumor, virally-infected, or MHC-I-mismatched cells and the organ in which these cells reside¹⁴⁵⁻¹⁴⁷. We did not explore the mechanism of NK trafficking in cardiac inflammation in this dissertation, and CFA may induce trafficking of NK cells to lymphoid nodes through the triggering of L-selection on NK cells¹⁴⁸. Any studies would be better served in T-cell adoptive transfer-induced EAM to separate out the confounding effects of CFA⁹⁴.

Cardiac NK cells upregulated levels of surface markers Ly49A/D and Ly49H, consistent with a mature and activated phenotype. Immature NK cells found in the bone marrow are largely Ly49 negative^{144,149}. A larger proportion of NK cells in the heart expressed CD27, expressed on a mature and cytotoxic NK subset thought to produce high levels of IFN γ , though in our model no difference was found between IFN γ production in CD27⁺ and CD27⁻ subsets in the heart (data not shown)^{150,151}. TRAIL, linked to the ability of NK cells to kill activated and virus-infected T cells, was also increased in the heart. Upregulation of TRAIL-death receptor (TRAIL-R2) on hepatitis virus B (HBV)-specific CD8⁺ T cells was required for NK mediated killing¹⁵². CD69, along with

increased IFN γ production, is one of few phenotypic markers that can distinguish activated pancreatic NK cells from diabetic NOD mice compared to pancreatic NK cells from diabetes-free strains¹⁵³.

NKG2D, surprisingly, was the only significantly downregulated marker queried on cardiac NK cells. NKG2D is a potent activation receptor also expressed on NKT, and $\delta\gamma$ T-cells and recognizes a subset of stress-induced MHCI-like molecules¹⁰⁴. Dysregulation of NKG2D has been implicated in the breakdown of self-tolerance in multiple autoimmunity models^{154,155}. Prevention of diabetes in NOD mice is possible by administering anti-NKG2D antibodies, preventing NK cells from recognizing the ligand Rae1 on pancreatic islet cells¹⁰⁶. Expression of NKG2D on CD4+ cells is correlated with disease severity in Crohn's patients and is enriched in the synovial fluid in RA. In all these studies, total NKG2D was targeted^{109,120}. Therefore, the functional role of NKG2D on NK cells specifically has not been determined in autoimmune models, and in our model of EAM, the downregulation of their expression indicates it may play a minimal role.

Besides their cytotoxicity potential, NK cells modulate the immune environment by the secretion of cytokines. NK cells may be functionally polarized by the same cytokine profiles that alter T-cell phenotypes^{156,157}. IFN γ is the prototypical cytokine produced by activated NK cells. IFN γ is induced largely by IL2, though secretion can also be triggered by IL1, IL12, IL18, and TNF α ¹⁵⁸. However, NK cells can be induced to express Th2 type cytokines IL5 and IL13 through IL4 and also by IL12 in IFN γ -deficient mice^{159,160}. NK cells in the heart during the peak of EAM produced IFN γ as their principal cytokine.

In this dissertation, we depleted NK cells by the administration of ASGM-1 antibody which left the NKT population intact in both naïve and inflamed animals. Thus, unlike many studies targeting NK1.1 expressed on both NK and NKT cells, we do not attribute alterations in phenotype to changes in the NKT compartment, though they also have been shown to have immune-modulatory roles in autoimmunity and inflammation. ASGM-1 treatment did, however, affect basophil populations (see Chapter 3). Surprisingly, the multiple injections of polyclonal rabbit IgG, as evidenced by the rabbit isotype controls, did not have any effects on disease severity, whether in positive manner by mimicking IVIG treatment as seen in rat isotype control antibodies or in a negative manner through the development of anti-rabbit IgG antibody complexes.

The depletion of NK cells led to increased inflammation and collagen deposition in the heart during in the inflammatory stages of EAM. As we did not examine the animals past day 21 of disease, the effects of NK cells on DCMI are unknown. ASGM-1 treated animals had decreased LV ejection fractions compared to control animals ultimately due to alterations in LV end systolic dimensions (LVESDs) with no changes in the LV end diastolic dimensions (LVESDd) seen of a dilated phenotype^{118,122}. Furthermore, while there were significantly drops in the cardiac output, none of the animals had the drastic reduction seen in mouse models of heart failure¹⁶¹⁻¹⁶³. We attribute the changes in systolic dimensions to the increased inflammation and collagen levels limiting the ability of the heart to contract. It is certainly possible that NK cells play a role in the development of DCMI given the increased collagen seen this early timepoint, but assessment of the mice at day 42 or later after the peak of inflammation subsides would be necessary.

While the cardiac NK cells profiled had increased expression of perforin and granzyme-B, their absence in the heart led to increased collagen deposition in the heart during myocarditis. Studies have indicated that NK cells can protect against the development of liver fibrosis by the specific targeting of activated hepatic stellate cells (HSCs) in 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), carbon tetrachloride and HCV models^{121,123,124}. Therefore, we hypothesized that NK cells limited fibroblasts through the killing of activated fibroblasts before collagen could be deposited. However, in series of co-culture experiments, we did not find that the co-culture of NK cells with activated fibroblasts resulted in any increase in death when compared to untreated fibroblasts.

While it would be ideal to have used activated NK cells directly from the heart of animals undergoing EAM, this is not feasible given the relatively small number of NK cells found in the heart during disease compared to the numbers required for an *in vitro* culture. We instead used polyI:C activated NK cells, known to increase their cytotoxicity potential, as an alternative⁹⁹. Both naïve and activated cells, though the latter population had upregulated expression of activation markers NKG2D and CD69, they also did not selectively target activated fibroblasts. Also, given the organ-specific properties of NK cells, it is likely that the mechanism of protection against fibrosis in the liver, associated with NKG2D expression, may widely differ from inflammatory cardiac NK cells^{107,108}.

CHAPTER IV: EOSINOPHILS ARE REQUIRED FOR NK-MEDIATED PROTECTION OF EAM

INTRODUCTION TO THE INTERPLAY OF EOSINOPHILS AND NK CELLS IN EAM

Biology and Development of Eosinophils

Eosinophils are innate granulocytes that comprise 1-3% of circulating peripheral blood cells¹⁶⁴. They are short-lived, with a lifespan of approximately 8 – 18 hours in the periphery. Upon migration into the tissue through diapedesis, eosinophils have a lifespan of 2-5 days depending on the inflammatory state of their microenvironment and up to 14 days *in vitro*¹⁶⁵. Most eosinophils will traffic to the digestive system, where they will line the lamina propria of the gastrointestinal tract under the control of eotaxin-1 (CCL11), though not eotaxin-2 (CCL24)¹⁶⁶. Under steady state conditions, eosinophils will also largely home to the mammary gland, thymus, and uterus, the latter being influenced of estrogen¹⁶⁷.

Eosinophils develop in the bone marrow from common myeloid progenitor (CMP) cells derived from multi-potent hematopoietic stem cells (HSC). These CMP cells give rise to a myeloblast believed to be the common progenitor for both basophils and eosinophils. From there, development into an immature eosinophil is regulated by transcription factors PU.1, GATA-1, and C/EBP¹⁶⁸. Mice lacking the GATA-1 promoter fail to develop mature eosinophils, though other cell lineages are unaffected¹⁶⁹.

Maturation and trafficking from the bone marrow in the periphery is under the control of IL5, with additive effects from GM-CSF and IL3^{170,171}. Intravenous injection of IL5 leads to high levels of peripheral eosinophils, a phenotype that is mirrored in multiple IL-5 transgenic models^{172,173}. IL5-deficient mice have severely reduced numbers of eosinophils and limited response to allergic or asthmatic stimuli¹⁷⁴.

Eosinophils are known for the tissue injury they can cause upon the release of their pre-formed granules of cationic and enzymatic proteins. Of these, major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) are the best characterized. Administration of purified MBP, EPO, and ECP to heart, brain, tumor, and epithelial tissue leads to cell damage and tissue death¹⁷⁵⁻¹⁷⁷. ECP has both cytotoxic effects by causing the formation of pores in target cells and non-cytotoxic effects in the suppression of T and B cells responses, degranulation of mast cells, and stimulation of mucus production in airway epithelium¹⁷⁸. MBP induces mast cell and basophil degranulation while affecting smooth muscle contraction and nerve plasticity^{179,180}.

Eosinophils can also secrete a number of cytokines such as IL2, IL4, IL13, and IL5, that can ultimately affect T-cell activation, differentiation, and proliferation¹⁸¹. They also can regulate Th1 differentiation through the release of indoleamine 2,3-dioxygenase (IDO), whose end-product, kynurines (KYN), promotes the apoptosis of Th1 cells¹⁸². There have also been reports that eosinophils can serve as antigen-presenting cells (APCs) under certain disease conditions such as asthma¹⁸³⁻¹⁸⁵. Eosinophils have also been shown to be required for repair of muscle injury and in the development of certain adipose cells through the recruitment of progenitor populations^{186,187}. Altogether, the mechanisms by which eosinophils modulate the immune system and local tissue environments, whether pathogenic or protective, can vary widely with each disease and should be determined on a case-by-case disease basis.

Eosinophils traffic to and infiltrate sites of injury and inflammation in response to a wide variety of signals. While IL5 appears to be the main controller of egress from the

bone marrow, the best known local positive chemotactic mediators include CCL11, CCL24, and CCL26 (human only), IL33, and prostaglandins^{168,170}. Conversely, they are negatively regulated through chemokines CXCL9 and CXCL10, which are recognized through receptors CXCR9 and CCR3¹⁸⁸⁻¹⁹⁰. Eosinophils pre-treated with CXCL9 and CXCL10 fail to respond to CCL11 and do not traffic to the lung when transferred into animals undergoing ovalbumin (OVA)-mediated models of asthma.

Eosinophils in asthma models have perhaps been best studied due to the close association between Th2 pathways and eosinophilic processes, though no single mediator of trafficking and accumulation has been determined. CCL11 and CCL24, traditionally upregulated by Th2-related cytokines IL4 and IL13, are major mechanisms of eosinophilic trafficking – eotaxin receptor (CCR3)-deficient mice display severely depressed levels of eosinophils in the lung and bronchial tubes in response to respiratory challenge of OVA following subcutaneous OVA-sensitization¹⁹¹. Only a limited reduction was seen, however, when CCR3-deficient mice were sensitized systemically¹⁹². Other studies have shown vital roles for various adhesion factors expressed on the surface of eosinophils in their entry into inflammatory sites – very late activation antigen 4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) expression on eosinophils is required for their infiltration during antigen-induced bronchial hypersensitivity¹⁹³⁻¹⁹⁵.

Interactions between eosinophils and their closely-related counterparts, mast cells and basophils, have also been major mechanisms of eosinophil regulation during disease. Certainly, all three are major components of most allergic inflammatory processes and express many of the same receptors and cytokines, such as IgG (FcγRII/CD32) and IgA (FcαRI/CD89) receptors and IL4, IL5, and IL13¹⁹⁶. All also express the IgE

receptor FcεR1, although eosinophils express this to lesser extent. Furthermore, eosinophil MBP can trigger degranulation of mast cells and basophils that may, in turn, result in the release of eosinophilic activation signals.

It has recently been shown that ILC2 cells residing in peripheral tissues are required for the maintenance of eosinophils^{131,132}. These cells are responsible for the maintenance of serum IL5 responsible for the egress of eosinophils out of the bone marrow. IL33 is a potent stimulator of ILC2s, and treatment of naïve mice with IL33 induces pericarditis¹⁶³. Stimulation of ILC2s in lung helminth infections results in the upregulation of both IL5 and IL13, inducing the expression of eotaxins and adhesion molecules on endothelial cells. This ultimately results in the onset of eosinophilia in the lung tissue¹³².

Hypereosinophilic syndrome and secondary presentations

Eosinophilia is defined as the abnormal enrichment of eosinophils in the periphery blood¹⁹⁷. This can be divided into two clinical subtypes: mild eosinophilia at less than 1.5×10^9 cells per mL but higher than normal blood eosinophilic counts and marked eosinophilia at greater than 1.5×10^9 cells per mL. Eosinophilia is a characteristic secondary feature of multiple disorders and infections and often occurs in selected tissues where inflammation is occurring¹⁹⁷. Tissue eosinophilia is characterized by the heavy presence of infiltrating eosinophils and, oftentimes, the deposition of eosinophilic granule proteins such as MBP^{198,199}.

Allergic reactions make up 80% of patients presenting with eosinophilia in the western hemisphere, with helminth infections comprising the majority of the remaining

20%²⁰⁰. In both these cases, eosinophilia is attributed to the increased in Th2 cells and subsequent secretion of eosinophilic chemotaxis-associated cytokines IL4, IL5, and IL13^{201,202}. In a small minority of cases, the underlying cause of elevated eosinophils are unknown, resulting in a diagnosis of idiopathic eosinophilia.

Hypereosinophilic syndrome (HES), a subset of idiopathic eosinophilic pathologies, is characterized by an eosinophil-dominant infiltration where eosinophils counts are higher than 1500/mm³ in tissue and/or persistent eosinophilia of 1.5×10^9 /L blood for more than six months²⁰³. HES may or may not be associated with specific organ damage and patients should first be examined to ensure that this eosinophilia is not secondary to parasitic infections, chronic eosinophilic leukemia (CEL), allergic reactions, or collagen vascular diseases (Table 5). CEL remains the most difficult to distinguish from HES due to significant overlap in clinical presentations, though the presence of *FIP1L1-PDGFR*A mutations associated with CEL can help differentiate diseases^{204,205}.

Clinical presentations of HES vary widely. A subset of patients may go on to develop significant tissue injury mediated by the release of eosinophilic granules containing EPO, ECN, MBP, and ECP.^{206,207} This is frequently seen in the heart, lungs, and skin. However, eosinophilic injury may also target the nervous system and the digestive tract. It is unknown what triggers the release of these granules in some patients, but not others²⁰⁸. Animal models of HES are limited, though HES may be induced by transgenic expression of IL5 under the CD2 or CD3 promoter^{209,210}.

The first line of therapeutic intervention for symptomatic HES is corticosteroids. In non-symptomatic patients, no therapy is required and patients are closely monitored for signs of tissue injury. The majority of patients respond to steroid treatment and may

be slowly weaned off as eosinophil levels decrease. If unresponsive to corticosteroids, the addition of interferon-alpha has proven beneficial²⁰⁶⁻²⁰⁸. The use of an anti-IL5 antibody known as mepolizumab as an alternative to corticosteroids has been met with limited success²¹¹.

Eosinophils in Autoimmunity

The composition of local infiltration in inflammatory disorders often determines the prognosis of the patient^{212,213}. In the case of eosinophils, a strong correlation between cellular frequency and poor clinical outcomes are reported frequently in both asthma/allergy and autoimmune models²¹⁴⁻²¹⁶. Thus, clinical necrotic eosinophilic myocarditis is strongly associated with the most severe pathology among the multiple myocarditis subsets. This subset is not associated with a peripheral eosinophilic disorder, but can develop unexpectedly from yet-unidentifiable triggers. Patients typically develop severe cardiac eosinophilic infiltration, elevated cardiomyocyte death, and intraventricular thrombi^{213,217-219}.

Necrotizing eosinophilic myocarditis is characterized by extensive cardiac eosinophilic infiltration, pronounced cardiomyocyte death, formation of intraventricular thrombi, and a case fatality rate of 50%.^{213,218-222} Similar correlations between eosinophil frequency in the infiltrate and poor clinical outcomes have been reported in other chronic inflammatory disease models, including asthma, inflammatory bowel disease (IBD), and experimental autoimmune encephalomyelitis (EAE).^{215,216,223}

Recent studies have demonstrated that infiltrating eosinophils significantly

increased disease severity in CBV-induced myocarditis when mice were injected with IL33. Administration of soluble ST2, an IL33 receptor decoy, prevented eosinophilia and significantly reduced viral myocarditis without altering viral burden¹⁶³. Similarly, IFN γ ^{-/-} IL17A^{-/-} mice display massively inflamed hearts and up to 50% fatality by day 21 of EAM, a phenotype that is reversed in the absence of eosinophils by crossing these animals to dblGATA1 mice²²⁴. Furthermore, eosinophilic infiltration in clinical myocarditis is strongly associated with negative clinical outcomes, supporting our findings.²¹⁷⁻²²⁰

Interplay of Eosinophils and Natural Killer Cells

NK cells are not major producers of eosinophilic mediators, with the exception of IL5. IL5, as a major mediator of eosinophil egress from the bone marrow into the peripheral blood, is a potent eosinophil chemoattractant and has been shown to be secreted by NK cells in asthma^{225,226}.

Depletion of NK cells using NK1.1 and ASGM-1 antibodies decreased eosinophils in the bronchoalveolar lavage fluid (BAL) prior to challenge with OVA, this being attributed the lack of IL5 from NK cells²²⁷. Another study showed that in mice were challenged with OVA, treatment of mice with NK1.1 resulted in the increased of eosinophils in the BAL, but further dissection of this using CD1d1-deficient mice indicated this was due to effects from the NKT compartment^{226,228}.

Recently, studies have shown that NK cells may directly induce apoptosis in eosinophils. Awad *et al* showed that NK cells isolated from human healthy PBMCs induced the activation and apoptosis of eosinophils as measured by CD62L and CD69

when co-cultured together *in vitro*. Apoptosis was initiated due to increases in reactive oxygen species (ROS) in eosinophils and cell death could be mitigated through the use of mitochondrial inhibitors rotenone and antimycin²²⁹.

Barnig *et al* demonstrated that NK cells from asthmatic patients were increased and more highly activated in the blood than those from healthy donors. NK cells isolated from asthmatic patients expressed increased levels of CD69, NKG2D, and ALX/FPR2, a receptor for lipoxin A4 (LXA4). LXA4 is elevated in the lungs of asthmatics and administration limits bronchoconstriction. Furthermore, LXA4 decreased eosinophilic trafficking and also limits NK cytotoxicity. The addition of LXA4 to co-cultures of NK cells and eosinophils isolated from the blood of both healthy and asthmatic donors limited the level of eosinophilic apoptosis²³⁰.

RATIONALE

In the previous chapter, we delineated the ability of NK cells to alter the phenotype of EAM through the limitation of cardiac inflammation and reduction of cardiac output due to increased infiltration and collagen deposition in the myocardium. We determined that cardiac NK cells have high cytotoxicity potential through increased perforin and granzyme-B and expressed largely IFN γ , with limited utility of the Th2-associated cytokines IL5 and IL13. However, we showed that despite cytotoxic potential and indicators from liver fibrosis models, NK cells did not directly kill cardiac fibroblasts *in vitro*.

In this chapter, we aim to explore the mechanisms by which NK cells control disease pathogenesis. Given the broad range of cell types that NK cells could potentially alter, we expected that depletion of NK cells might have a variety of effects on the cardiac infiltrate. We hypothesize that the depletion of NK cells would result in the expansion of CD4 T cells in response to immunization, specifically in the Th2 and Th17 components, given the IFN γ production from NK cells. Furthermore, this expansion in Th2 and Th17 cells would be the driver of pathogenesis through the modulation of monocyte populations, similar to other findings in our lab^{36,140,231,232}.

RESULTS

Eosinophils are increased in the absence of NK cells during EAM

To determine the mechanism of NK-mediated suppression of cardiac inflammation, we performed comprehensive flow cytometry to examine cardiac infiltrate changes during EAM following NK depletion. NK-depleted animals had increased SSC^{hi}CD45⁺ granulocytic cells (Figure 15A). We analyzed which granulocyte population was responsible for the escalation and found that SSC^{hi}Ly6G^{lo}SiglecF⁺ eosinophils increased 2-fold on day 14 and 10-fold on day 21 in NK-depleted animals compared to isotype control animals (Figure 15B – 15C). No changes were seen in SSC^{hi}Ly6G^{hi} neutrophils on day 14 and 21 (Figure 15D – 15E).

Eosinophils in the heart of ASGM-1 treated mice have an activated profile

We examined the phenotype of these infiltrating eosinophils to determine their maturation and activation status. MBP is largely post-transcriptionally regulated and is released into the tissue upon activation. Therefore, we stained paraffin-embedded cardiac tissue section with rat anti-MBP primary antibody (1:500) followed by donkey anti-rat IgG secondary antibody conjugated to Texas Red and counterstained samples with 4',6-diamidino-2-phenylindole (DAPI) to distinguish cell nuclei. ASGM-1 treated animals showed positive staining for MBP presence (Figure 16A). SiglecF and CD11b are markers of eosinophilic activation. In order to determine whether eosinophils in the heart at day 21 of EAM in NK-depleted animals represented an activated and deliberate subset compared to those in the periphery, we examined SiglecF and CD11b mean fluorescence

intensity (MFI) on cardiac and splenic Ly6G^{lo}SiglecF⁺ eosinophils and found that cardiac eosinophils had upregulated levels of SiglecF and CD11b (Figure 16B and 16C)

We also profiled the mRNA transcriptome of heart-infiltrating eosinophils at day 21 of EAM compared to spleen eosinophils. Consistent with an activated and mature phenotype, cardiac eosinophils downregulated mRNA levels of *epx* and *prg2* (Figure 17A and 17B). They also expressed more activation associated mediators IL1 β , CCL11, and IL6, and showed no changes in TGF β , IL4, IL13, CCL24, or IL10 (Figures 17C – 17J) by qPCR, normalized to levels of HPRT. In summary, eosinophils infiltrating the heart at day 21 of ASGM-1 treated mice represent a distinct and activated subset population compared to cells in the periphery.

NK depletion has no effect on mast cells or myeloid cells during EAM

The proportions of Fc ϵ RI α ⁺cKit⁺ mast cells remained unchanged in response to ASGM-1 treatment (Figure 18A). Along with this, levels of IL33 and ST2, known mast cell-derived mediators of eosinophilic accumulation, also remained unaltered (Figure 18B and 18C). However, basophil populations, consistent with published literature, were depleted in response to ASGM-1 treatment during EAM (Figure 18D). Therefore, although mast cells may play an undeniable role in many models of allergy, asthma, and autoimmunity, it is unlikely that mast cells play a major role in the modulation of disease pathogenesis or eosinophilic accumulation in response to NK depletion in this model of myocarditis.

Myeloid-derived populations generally make up the bulk of infiltrating cells in the heart during EAM and are major players in the modulation of disease. We profiled

total CD11b⁺ monocytes from isotype control and ASGM-1 treated mice with EAM and saw that there was no alterations in proportions of total monocytes (Figure 19A). Furthermore, we examined monocyte subsets based on Ly6C expression and found no changes in inflammatory Ly6C^{hi} or resident-type Ly6C^{lo mid} monocyte proportions (Figure 19B – 19C). We also looked at levels of CD11c⁺ dendritic cells, as these cells are major mediators of autoimmunity through the presentation of self-antigen to T cells, but no changes were found (Figure 19D). It is unlikely that the shifts in disease severity due to NK depletion are from alterations in the myeloid compartment.

NK depletion increases Th2 and Th17 proportions at later stages of EAM

EAM is a T-cell mediated disease that can potentially be influenced by antibody deposition so we examined proportion of infiltrating B cells and T cells at day 21 of EAM in ASGM-1 treated and isotype control mice. We saw no shifts in the proportions of total CD19⁺ B cells or CD3⁺ T cells (Figure 20A and 20B). We further examined CD4⁺ and CD8⁺ T cell compartments and noted that these were also unaltered by NK depletion (Figure 20C and 20D).

As EAM severity can be influenced by shifts in the polarization of CD4 populations,^{36,216,233} we examined how depletion of NK cells affected Th1, Th2, and Th17 populations on days 14 and 21 of EAM in WT heart by flow cytometry. No changes in IFN γ ⁺ Th1 cells were found (Figure 21A). However, IL13⁺ Th2 and IL17A⁺ Th17 cells as a fraction of total CD3⁺CD4⁺ cells significantly increased at day 21 of EAM (Figure 21B and 21C). It should be noted that due to known difficulties with IL4 antibody staining, IL13 was used as a surrogate marker for Th2 polarization.

The finding of elevated Th2 and Th17 populations at day 21 of EAM was supported by examining cardiac mRNA expression of Th-associated cytokines on day 21 of EAM. qPCR data showed significant increases in Th2-associated *il5* and Th17-associated *il6* in the hearts of ASGM-1 antibody treated mice (Figure 22A and 22E). Interestingly, we observed decreased IL17A in contrast to increased Th17 CD4⁺ cells (Figure 22C). This likely reflects the loss of NK-sourced IL17A. We found no changes in levels of *ifng*, *il10*, *il13*, and *il12b* (comprising both IL23 and IL12) (Figure 22B – 22G). Thus, NK depletion increased Th2 and Th17 associated cytokines on day 21 of EAM.

Eosinophils are essential for increased myocarditis severity in the absence of NK cells

To establish if the greater EAM severity in NK-depleted animals depended on the influx of eosinophils in the heart, we depleted NK cells from eosinophil-deficient Δ dblGATA1 mice.¹⁶⁹ We used the same protocol as described previously for NK cell depletion and sacrificed the mice on day 21. Due to the lack of eosinophils in Δ dblGATA1 mice, NK depletion had no effect on eosinophil numbers (Figure 23A) in contrast to the WT phenotype (Figure 23C and 23D). This lack of eosinophils resulted in comparable EAM levels between NK depleted and isotype Δ dblGATA1 groups (Figure 23B). Depletion of NK cells from Δ dblGATA1 mice had no effect on heart function and cardiac fibrosis compared to WT animals undergoing EAM (Figure 23C and 23D). Our results demonstrate from the first time that the absence of NK cells leads to more severe pathology through increased eosinophilic infiltration.

Th-dysregulation in the absence of NK cells is independent of disease severity

To determine if the alterations in disease severity from eosinophils in ASGM-1 treated mice were primarily due to changes in Th compartments and their associated cytokines, we profiled the whole heart tissue homogenates of Δ dblGATA1 for their expression of IFN γ , IL4, IL6, and IL17A mRNA at day 21 of EAM after isotype control or ASGM-1 treatment as surrogate indicators for Th populations. We show that in the absence of eosinophils and changes in disease severity, increases of IL4 and IL6, the major effector cytokines of Th2 and Th17 cells, are still seen (Figure 24A – 24D). Thus, shifts in Th populations still occur in NK depleted animals with limited changes to clinical severity in the absence of eosinophils.

NK cells prevent production of eosinophilic chemokines in the heart during EAM

To investigate the mechanism of eosinophil accumulation in the heart, we assessed the relative cardiac versus splenic expression of eosinophil-related chemokines in NK-depleted and isotype control animals during EAM. mRNA levels of *ccl11* (eotaxin-1) and *ccl24* (eotaxin-2), chemoattractants for eosinophils, were comparable on day 14 (Figure 25A and 25B); however, CCL11 was significantly greater in NK-depleted animals by day 21 (Figure 25C).

Chemokines CXCL9 (MIG) and CXCL10 (IP10) are known negative regulators of eosinophil trafficking²³⁴. On day 14, *cxcl9* was significantly decreased in the NK-depleted group with *cxcl10* trending (Figure 25D and 25E). Therefore, NK cells can control the expression of positive and negative regulators of eosinophilic trafficking in the heart during EAM.

NK cells control eosinophil-related chemokine expression in cardiac fibroblasts

NK cells themselves are not major producers of eosinophilic chemokines^{99,235,236} and with the exception of eosinophils, there were no changes in inflammatory populations during EAM. We have found recently that cardiac fibroblasts (CFs) are a major source of chemokines and cytokines during EAM.²³² Therefore, we investigated the ability of NK cells to control chemokine production from CFs. CFs and NK cells added (1:2 ratio) for 96 hours with IL4 (10ng/mL) to mimic an environment favorable for eosinophilic accumulation.

NK cells suppressed IL4-mediated CCL11 production from CFs (Figure 26A). Additionally, NK cells significantly increased expression of CXCL9 and CXCL10, two negative regulators of eosinophil trafficking (Figure 26B and 26C). This pattern was seen for other chemokines that promote trafficking of myeloid cells, T cells, and neutrophils including CCL2 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), and CXCL1 (KC), although these chemokines are associated with the positive regulation of eosinophil trafficking (Figure 26D–26G). IL5, a potent eosinophilic growth factor and chemoattractant, was produced by CFs in the presence of IL4, although levels did not change upon addition of NK cells (Figure 26H).

We further confirmed the ability of NK cells to downregulate CCL11 and CCL24 from resident fibroblasts *in vivo* by isolating CFs from NK-depleted and isotype control hearts on day 21 of EAM. mRNA expression of *ccl11* and *ccl24* were increased in the fibroblasts isolated from NK depleted animals when compared to rabbit IgG treatment (Figure 27A and 27B). Thus, NK cells directly control eosinophil-related chemokine

expression in cardiac fibroblasts *in vitro* and *in vivo*.

CCL11 and CCL24 are not required for NK-mediation of eosinophils

These chemokine data presented us with two non-exclusive mechanisms by which NK cells regulate eosinophil trafficking: a) suppressing CCL11/CCL24-mediated recruitment or b) increasing CXCL9/CXCL10-mediated inhibition of trafficking. We investigated these mechanisms independently. To address the hypothesis that NK cells downregulate eosinophil accumulation by suppression of eotaxins, we employed mice deficient in CCR3, the sole receptor of CCL11 and CCL24²³⁴. Depletion of NK cells in CCR3^{-/-} mice still resulted in the influx of eosinophils into the heart during EAM (Figure 28A and 28B), indicating that NK cells do not mediate eosinophil accumulation in the heart only through the eotaxin/CCR3 pathway.

IFN γ is not required for NK-mediation of eosinophils

To investigate our secondary hypothesis that NK cells suppress eosinophils by upregulating negative chemokines CXCL9 and CXCL10, we employed IFN γ ^{-/-} mice, as both chemokines are dependent on IFN γ . NK cells were unable to downregulate CCL11 and upregulate levels of CXCL9, and CXCL10 from IFN γ R1^{-/-} CFs *in vitro*, suggesting IFN γ as the major mediator (Figure 29A – 29C).

Furthermore, we compared levels of CCL11, CXCL9, and CXCL10 in WT and IFN γ ^{-/-} mice at day 21 of EAM. We saw significant decreases in the protein levels of CXCL9, though there were no changes in levels of CCL11 or CXCL10 in the hearts of IFN γ ^{-/-} animals, suggesting other regulators may be at play *in vivo* for CCL11 and

CXCL10 (Figure 30A – 30C).

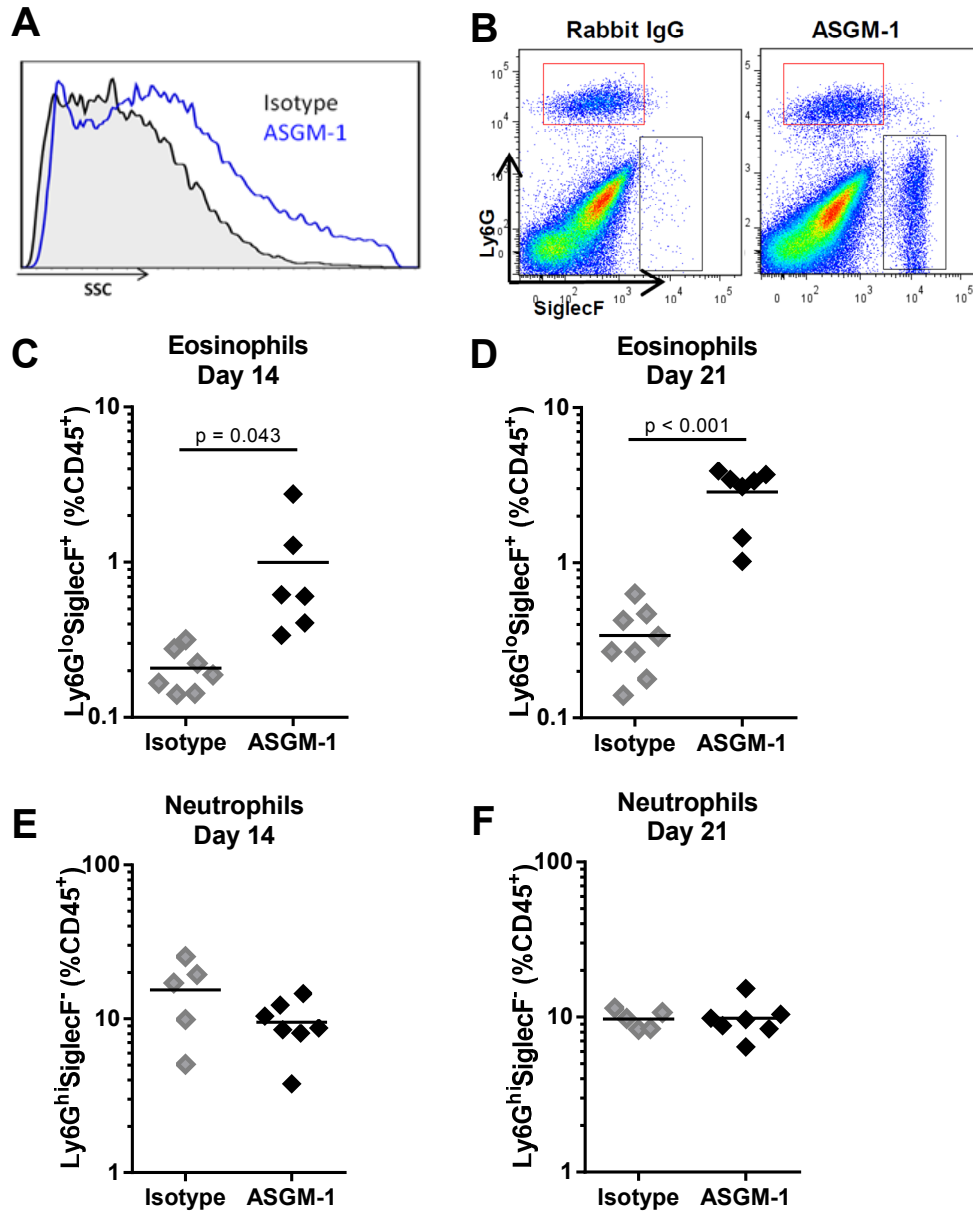
Finally, using $\text{IFN}\gamma^{-/-}$ mice to model the effects of diminished CXCL9, we depleted NK cells in $\text{IFN}\gamma^{-/-}$ mice and examined eosinophilic accumulation in the heart on day 21. Similar to the $\text{CCR3}^{-/-}$ mice, $\text{IFN}\gamma^{-/-}$ mice still continued to accumulate significantly more eosinophils in the heart in the absence of NK cells (Figure 31A). From our data employing the $\text{CCR3}^{-/-}$ and $\text{IFN}\gamma^{-/-}$ models, we conclude that neither mechanism is sufficient to mediate NK-mediated control of eosinophil in isolation. Overall, these results demonstrate that there may be multiple redundant chemokines responsible for cardiac eosinophilic accumulation during EAM.

FIGURES AND TABLES

Table 4. Diagnosis of Hypereosinophilic Syndrome (HES)^{201,203,207}

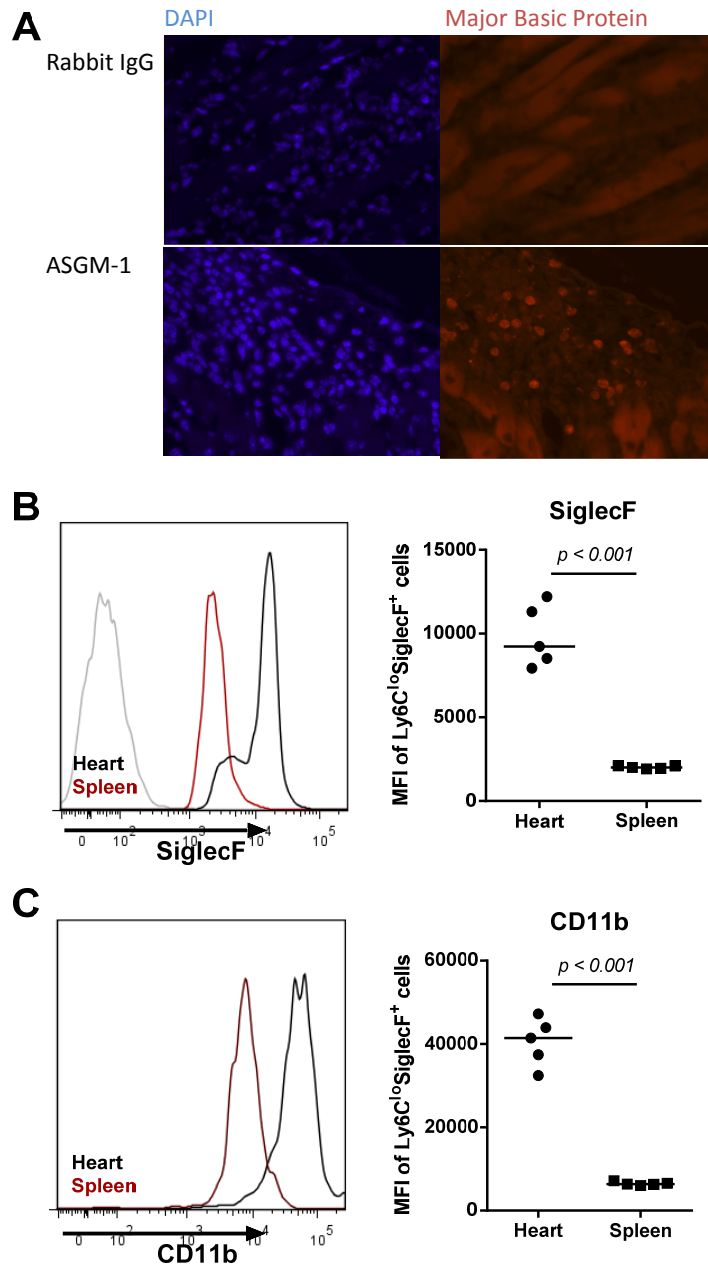
	Condition
1	Exclude patients with eosinophilia secondary to allergy, parasitic disease, infectious disease, and pulmonary diseases such as Loeffler's or hypersensitivity pneumonitis
2	Exclude patients with neoplastic disorders with secondary eosinophilia
3	Exclude patients with neoplastic disorders involving eosinophils
4	Exclude patients presenting with abnormal T-cell phenotypes
CEL	Conditions 1-4 have been met Myeloid compartment demonstrates clonal abnormalities Myeloblasts are > 2% in peripheral blood and between 5 and 19% in bone marrow
HES	Conditions 1-4 have been met and there are no myeloid abnormalities

Figure 15. NK cells suppress cardiac eosinophilic accumulation during EAM



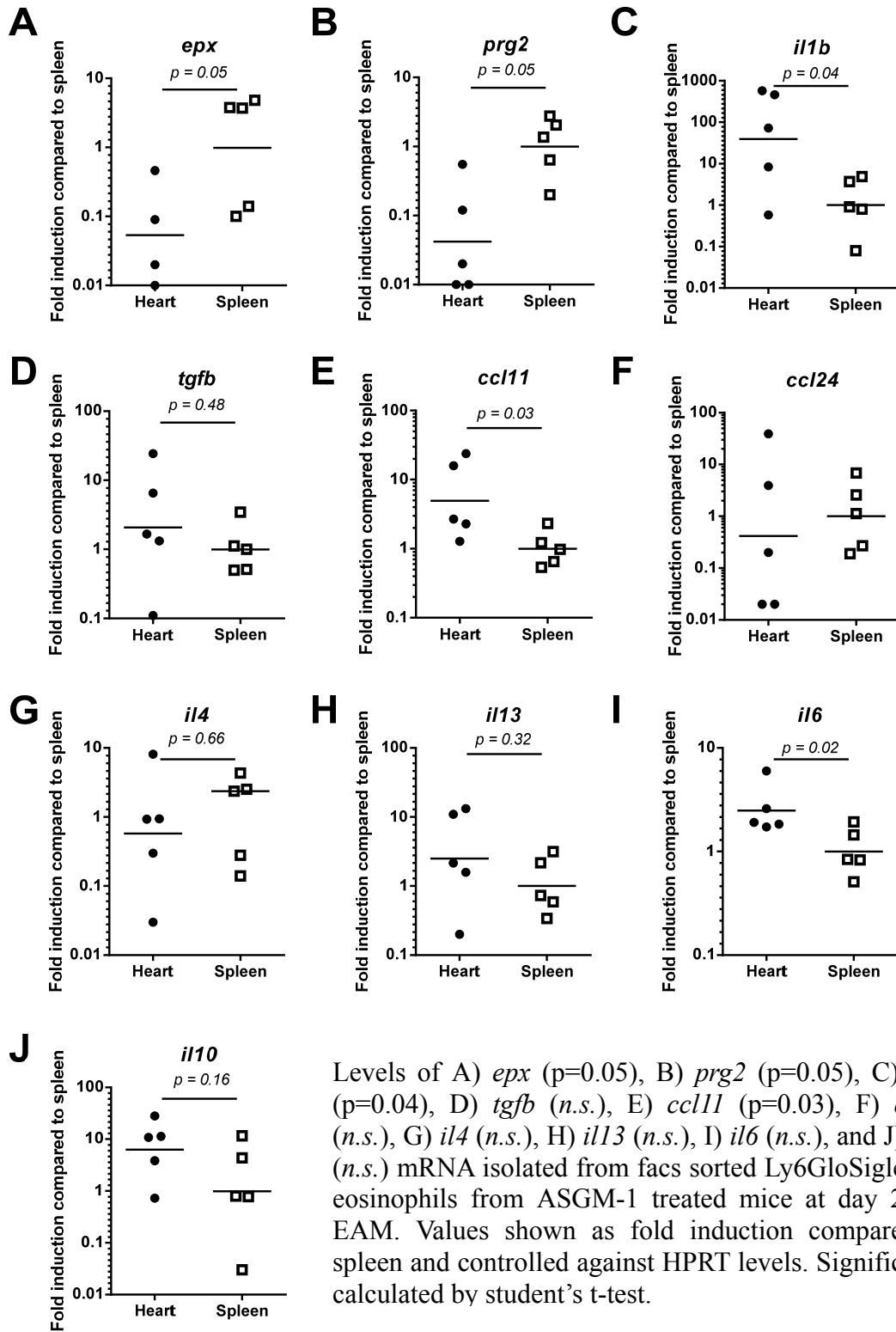
A) Representative side scatter histograms of isotype and ASGM-1 antibody treated animals at day 21. B) Representative Ly6G versus SiglecF bivariate plots at day 21 from viable CD45⁺ cells. C) Percentage of Ly6G^{lo}SiglecF⁺ eosinophils from cardiac CD45⁺ cells on D) day 14 ($p=0.043$) and E) day 21 ($p<0.001$) of EAM. Percentage of SiglecF⁻ Ly6G^{hi} neutrophils at F) day 14 (*n.s.*) and G) day 21 (*n.s.*). Significance calculated by unpaired *t*-test.

Figure 16. Increased eosinophils express high levels of MBP, SiglecF, and CD11b



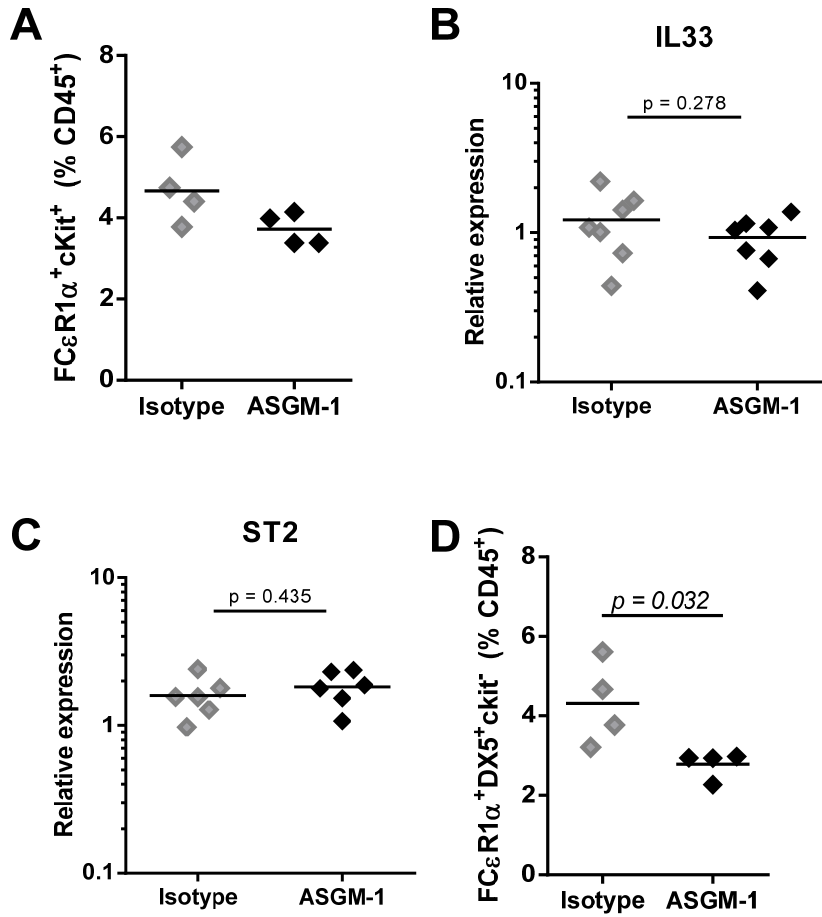
A) Immunofluorescence staining of paraffin embedded cardiac sections from isotype control and ASGM-1 treated mice on day 21 of EAM. Sections were stained with 1:500 rat anti-MBP primary or rat IgG antibody, 1:200 anti-rat donkey PE-Texas Red antibody, and DAPI. Relative mean fluorescence intensity (MFI) of B) SiglecF ($p < 0.001$) and C) CD11b ($p < 0.001$) expression on Ly6C^{lo}SiglecF⁺ eosinophils from the heart and spleen of isotype control and ASGM-1 treated animals at day 21 of EAM by flow cytometry. Statistics calculated by unpaired t-test.

Figure 17. Eosinophils in the heart of ASGM-1 treated mice have an activated profile



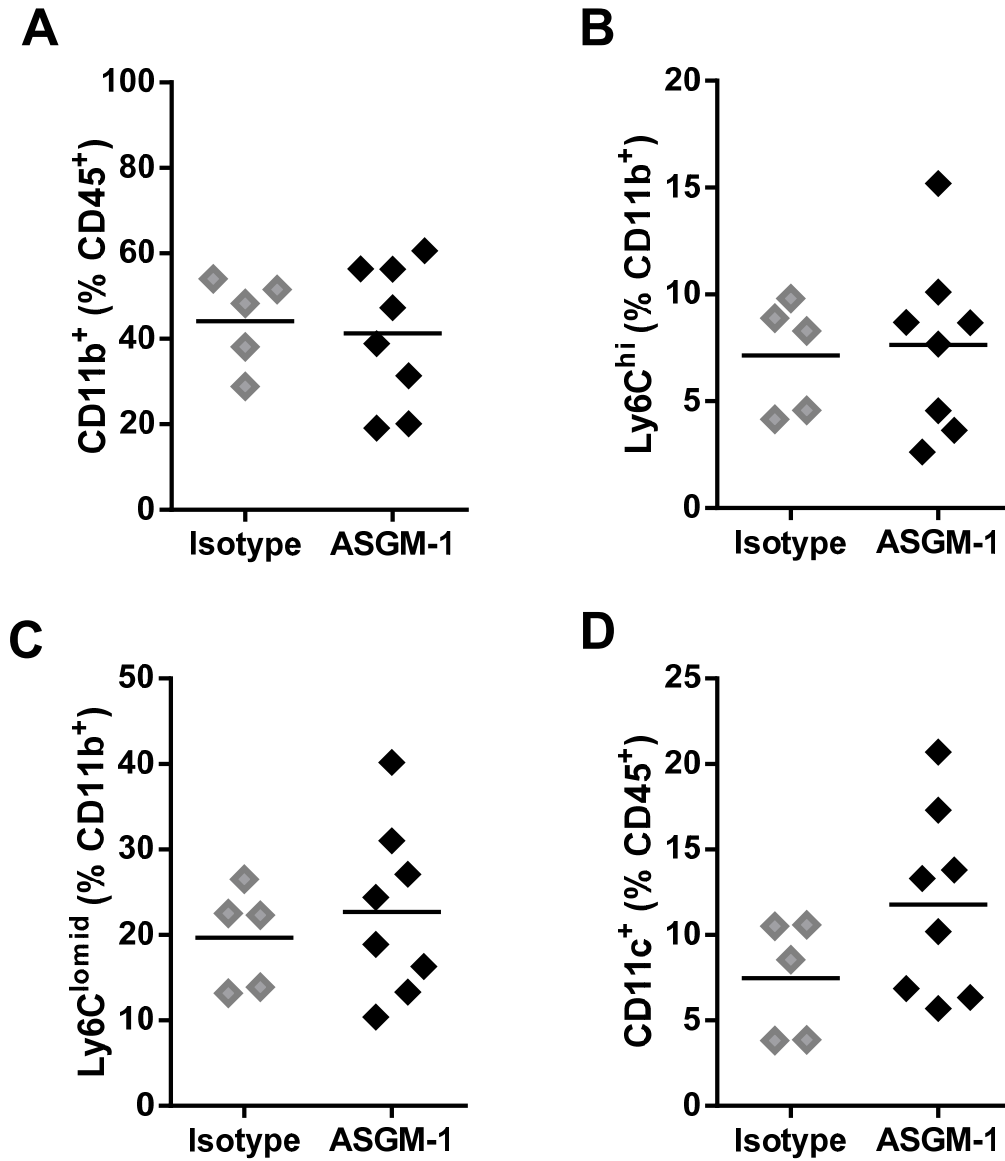
Levels of A) *epx* ($p=0.05$), B) *prg2* ($p=0.05$), C) *il1b* ($p=0.04$), D) *tgfb* (*n.s.*), E) *ccl11* ($p=0.03$), F) *ccl24* (*n.s.*), G) *il4* (*n.s.*), H) *il13* (*n.s.*), I) *il6* (*n.s.*), and J) *il10* (*n.s.*) mRNA isolated from facs sorted Ly6GloSiglecFhi eosinophils from ASGM-1 treated mice at day 21 of EAM. Values shown as fold induction compared to spleen and controlled against HPRT levels. Significance calculated by student's t-test.

Figure 18. Mast cells, and mast cell mediators are not altered in the absence of NK cells



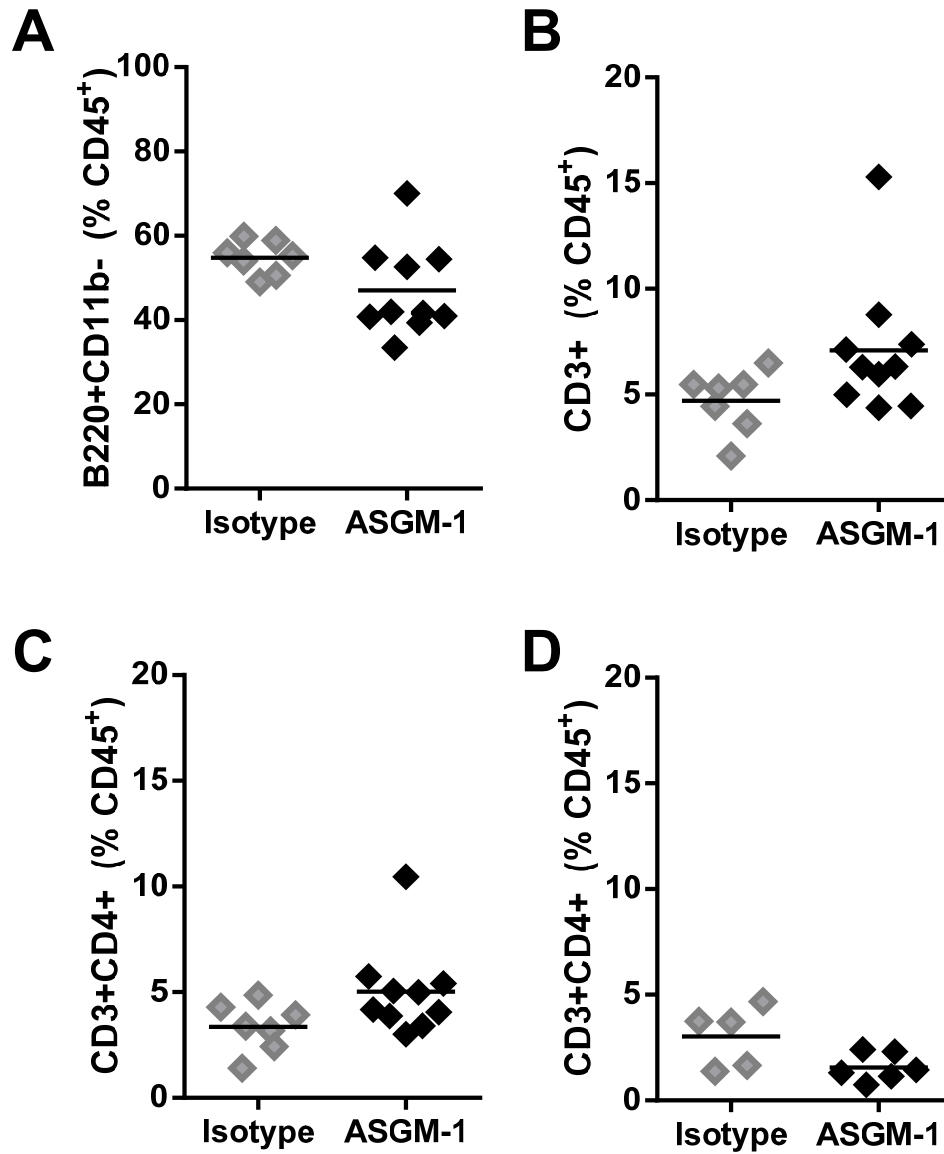
A) FCεR1α⁺cKit⁺ mast cells (*n.s.*) as a percentage of total CD45⁺ cardiac cells at da 14 of EAM in isotype and ASGM-1 treated WT animals. Relative mRNA levels of mast cell-associated mediators B)IL33 (*n.s.*) and C) ST2 (*n.s.*) in total heart homogenates at day 14 of EAM by qPCR normalized to levels of HPRT. D) FCεR1α⁺DX5⁺cKit⁻ basophils (*p*=0.032) as a percentage of total CD45⁺ cardiac cells at day 14 of EAM in isotype control and ASGM-1 treated animals. Significance determined by unpaired t-test.

Figure 19. Myeloid populations are unaffected by NK depletion during EAM



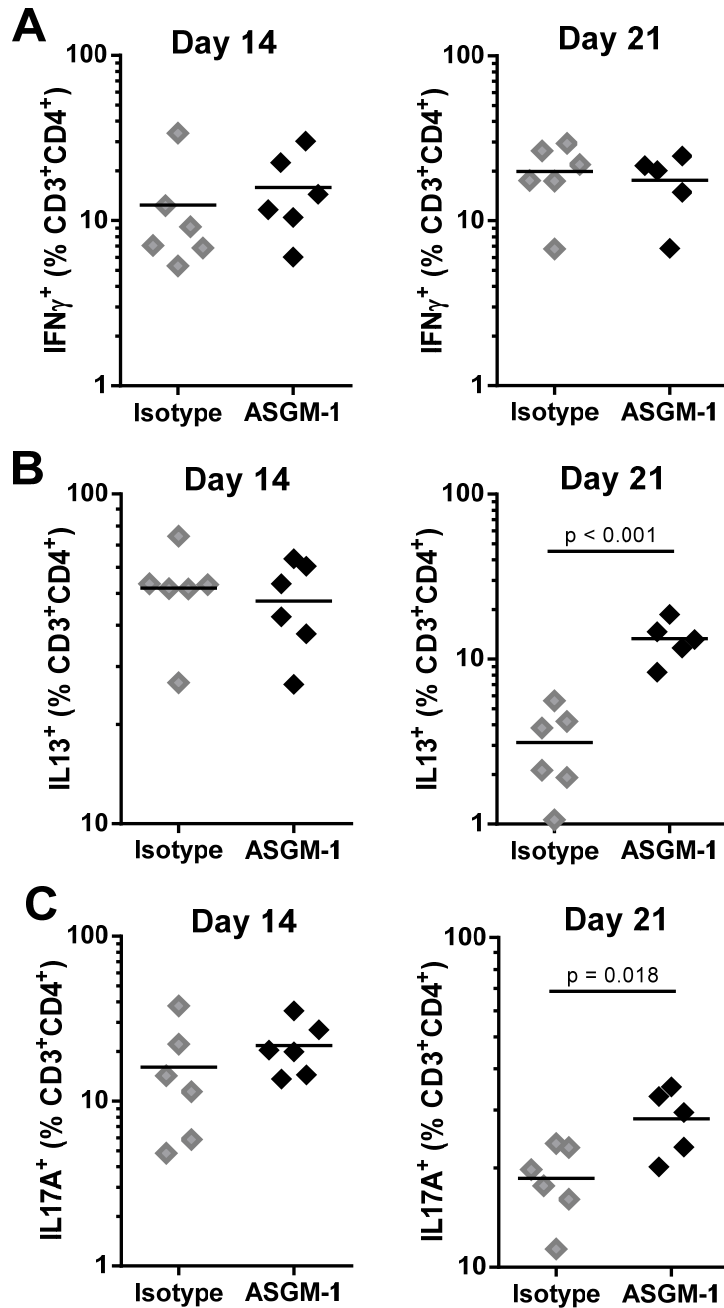
A) CD11b⁺ monocytes (*n.s.*) as a percentage of total CD45⁺ cardiac cells at day 14 of EAM in isotype and ASGM-1 treated WT animals. B) Ly6C^{hi} inflammatory (*n.s.*) and C) Ly6C^{lomid} resident (*n.s.*) subsets as a percentage of total CD11b⁺ cardiac monocytes. D) CD11c⁺ dendritic cells (*n.s.*) as a percentage of total CD45⁺ cardiac cells at day 21 of EAM in isotype control and ASGM-1 treated animals. Significance determined by unpaired t-test.

Figure 20. B cell and T cell proportions are unaffected by NK depletion during EAM



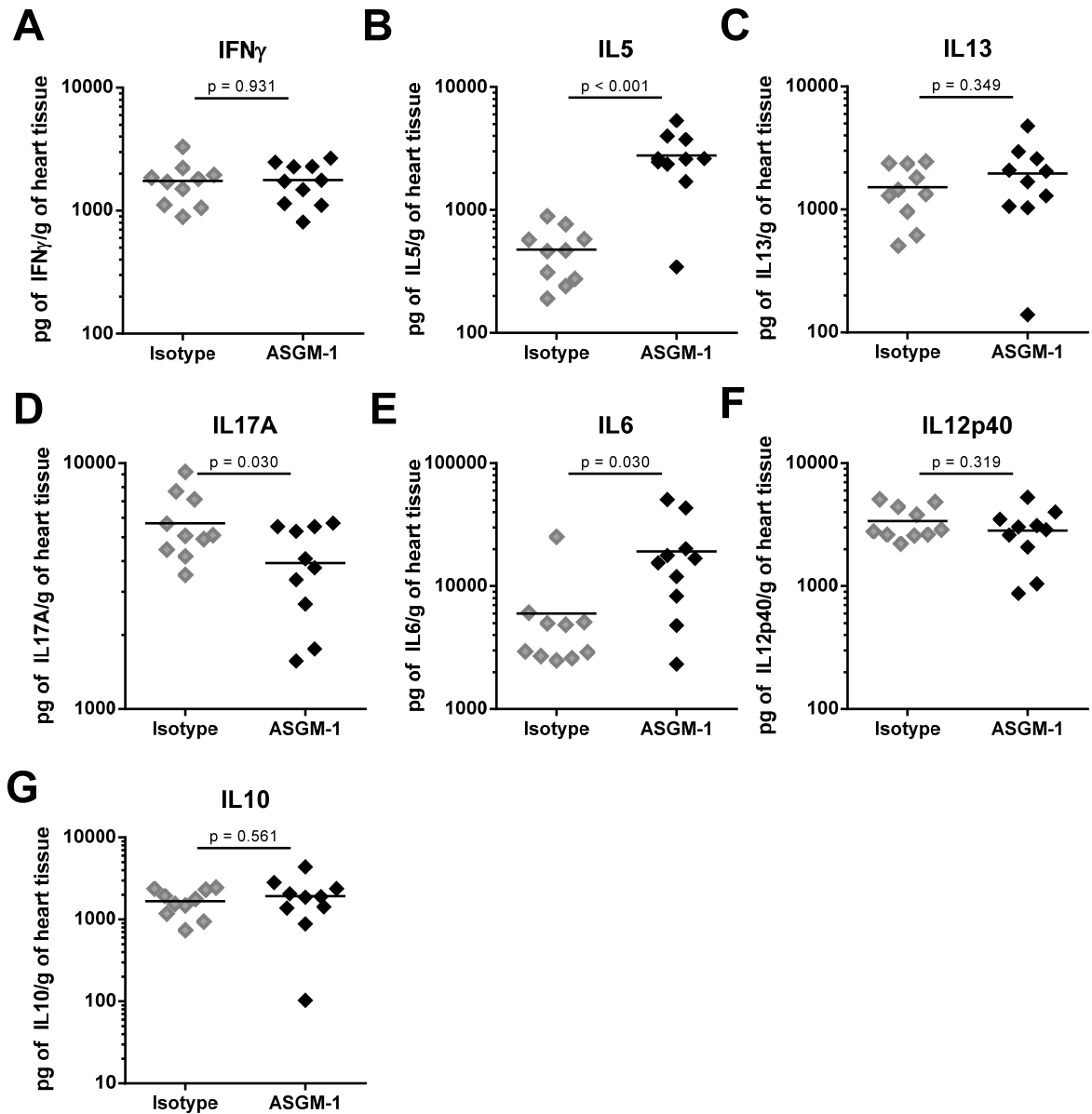
Lymphocyte percentages of A) B220⁺ B-cells (*n.s.*), B) CD3⁺ T cells (*n.s.*), C) CD3⁺CD4⁺ helper T cells (*n.s.*) and D) CD3⁺CD8⁺ cytotoxic T cells (*n.s.*) as a percentage of total CD45⁺ cardiac cells as a percentage of total CD45⁺ cardiac cells at day 21 of EAM in isotype control and ASGM-1 treated animals. Significance determined by unpaired t-test.

Figure 21. NK depletion increases Th2 and Th17 proportions at later stages of EAM



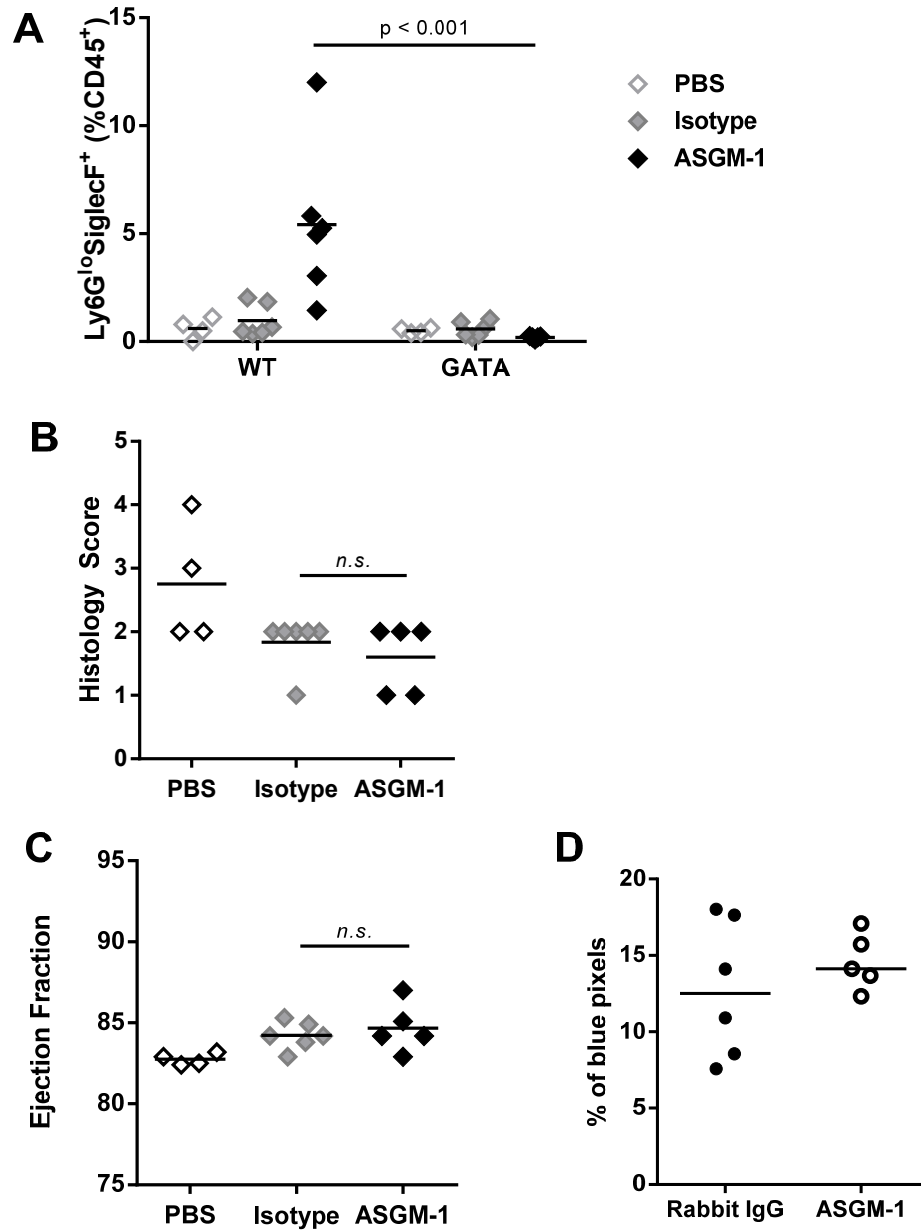
T-cell subset examination by intracellular cytokine staining for A) IFN γ (Th1), B) IL13 (Th2), and C) IL17A (Th17), as a proportion of cardiac CD3⁺CD4⁺ cells at day 14 (all *n.s.*) and day 21 of EAM following 4-6 hours of PMA and ionomycin stimulation with Golgistop. Significance calculated by unpaired *t*-test.

Figure 22. Day 21 cardiac cytokine levels reflect changes in Th profiles



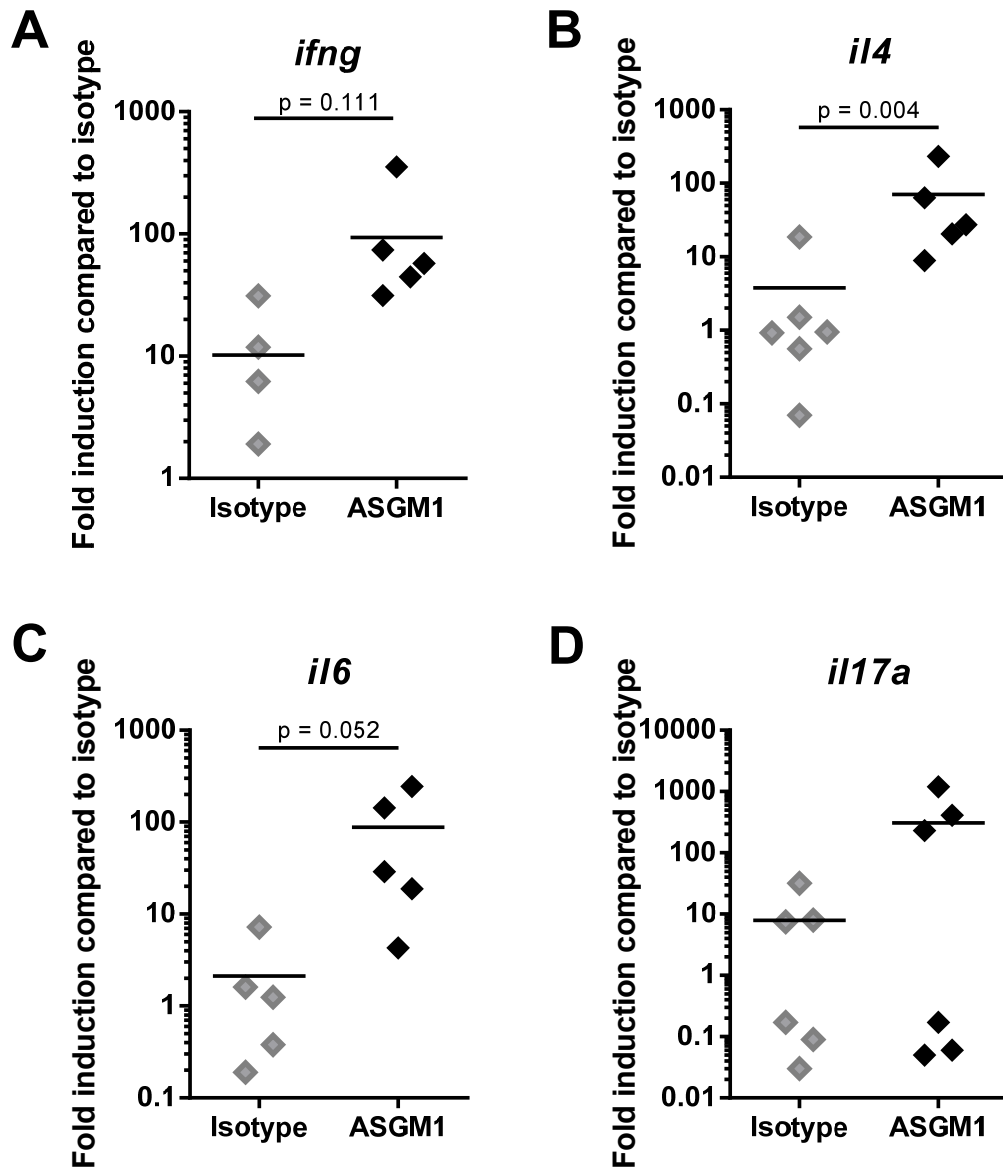
Protein analysis of cardiac tissue by LINCO at day 21 of EAM in isotype control and ASGM-1 treated animals of A) IFN γ (*n.s.*), B) IL5 ($p < 0.001$), C) IL13 (*n.s.*), D) IL17A ($p = 0.030$), E) IL6 ($p = 0.030$), F) IL12p40 (*n.s.*), and G) IL10 (*n.s.*). Significance determined by student's t-test.

Figure 23. Eosinophils are essential for increased myocarditis severity in the absence of NK cells



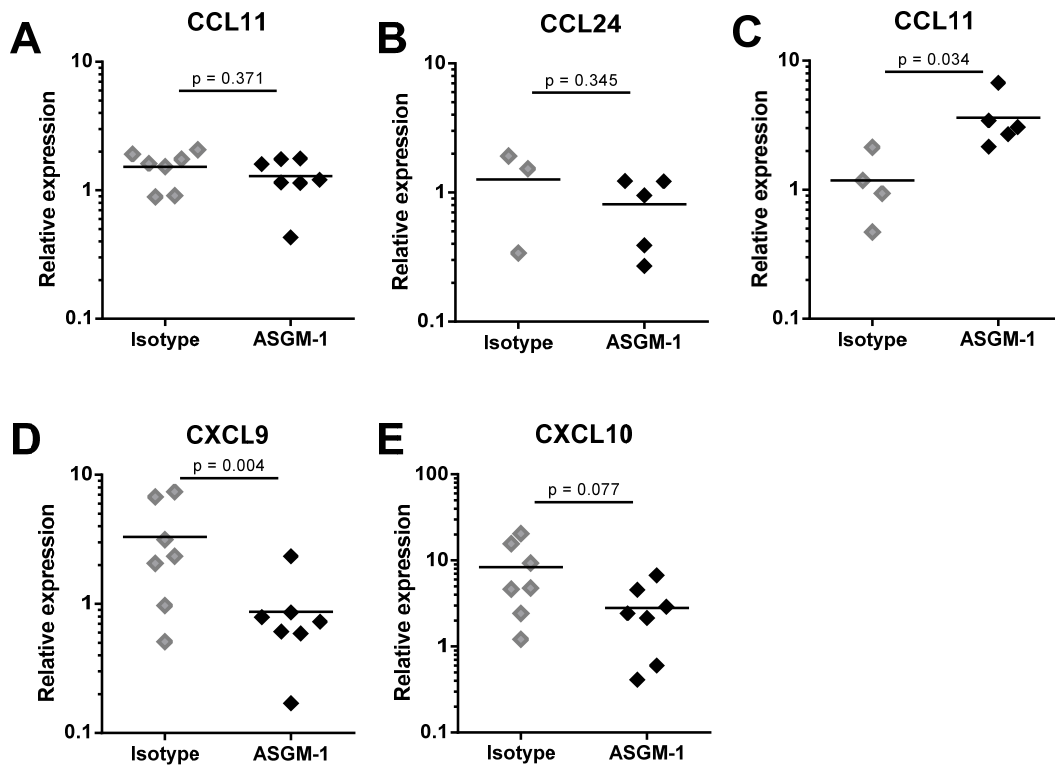
A) Percent of cardiac SiglecF⁺ eosinophils in WT and Δ dblGATA1 groups during EAM at day 21 (ANOVA $p < 0.001$) B) Histology scores of PBS, rabbit IgG, and ASGM-1 antibody treated Δ dblGATA1 animals at day 21 of EAM ($n.s.$). C) Ejection fraction ($n.s.$) measured by echocardiography at day 21. D) Percentage of fibrosis ($n.s.$) as measured by Masson's Trichrome staining of cardiac sections at day 21. Significance by ordinary one-way ANOVA with post-testing by Tukey's multiple comparisons test.

Figure 24. Th-dysregulation in the absence of NK cells is independent of disease severity



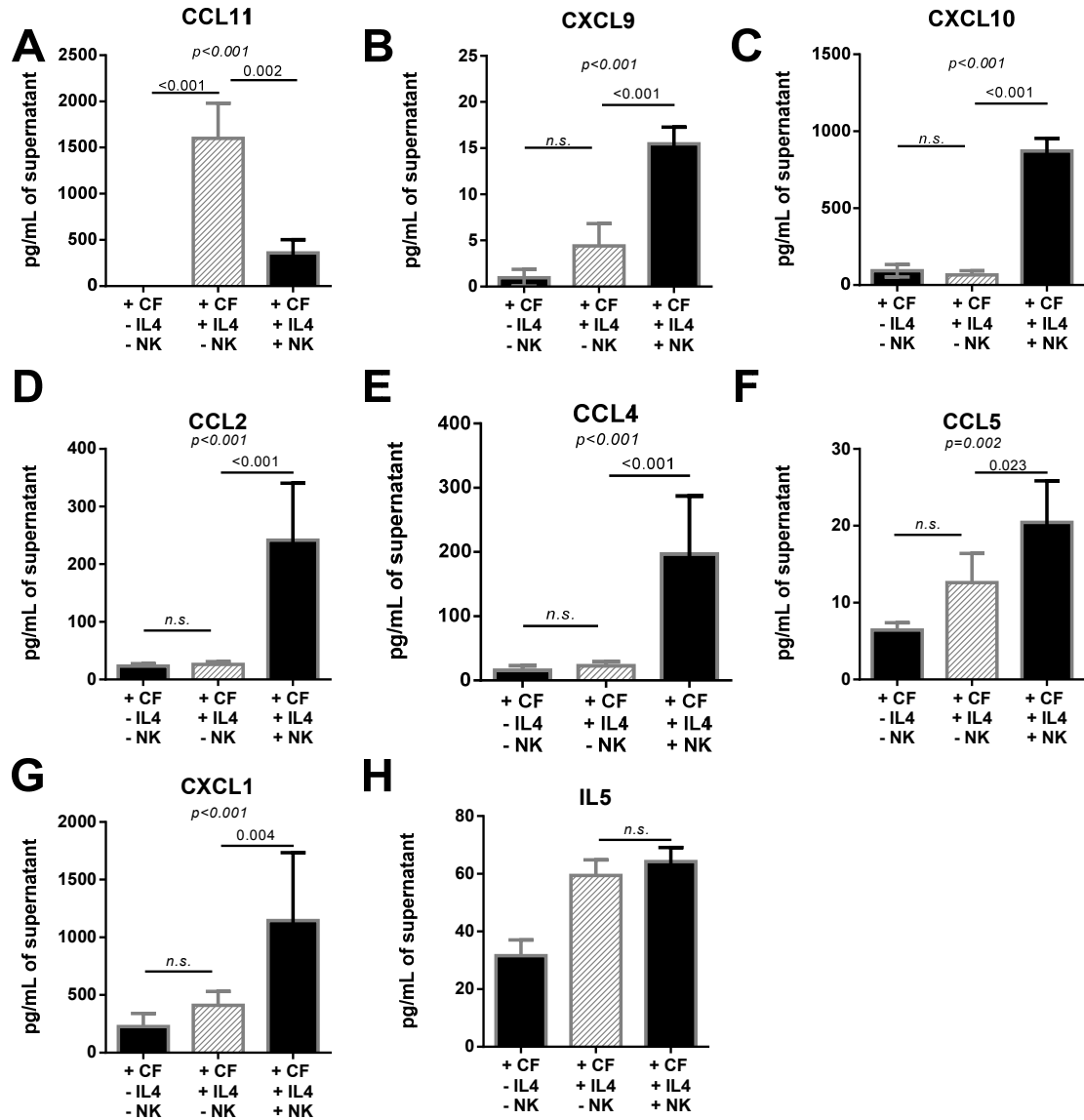
T-cell subset examination by qPCR for A) IFN γ (Th1) (*n.s.*), B) IL4 (Th2) ($p=0.004$), C) IL6 (Th17) ($p=0.052$), and D) IL17A (Th17) (*n.s.*) mRNA in whole heart tissue homogenates normalized against HPRT mRNA levels and shown as a function of fold induction against isotype control levels. Significance calculated by unpaired *t*-test.

Figure 25. NK cells alter levels of eosinophil-associated chemokines in vivo



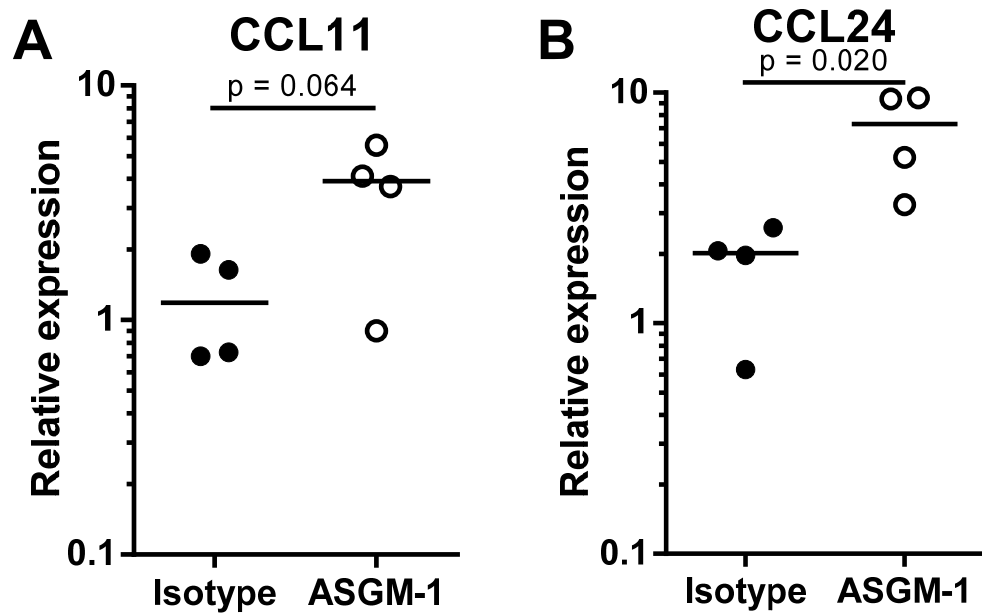
Levels of A) *ccl11* (*n.s.*) and B) *ccl24* (*n.s.*) mRNA at day 14 of EAM. C) CCL11 ($p=0.035$) at day 21 of EAM. D) *cxcl9* ($p=0.004$), and E) *cxcl10* ($p=0.077$) mRNA at day 14 of EAM in ASGM1- and rabbit IgG-treated animals measured by qPCR of cardiac tissue. Significance calculated by unpaired t-test.

Figure 26. NK cells alter levels of eosinophil-associated chemokines from CFs in vitro



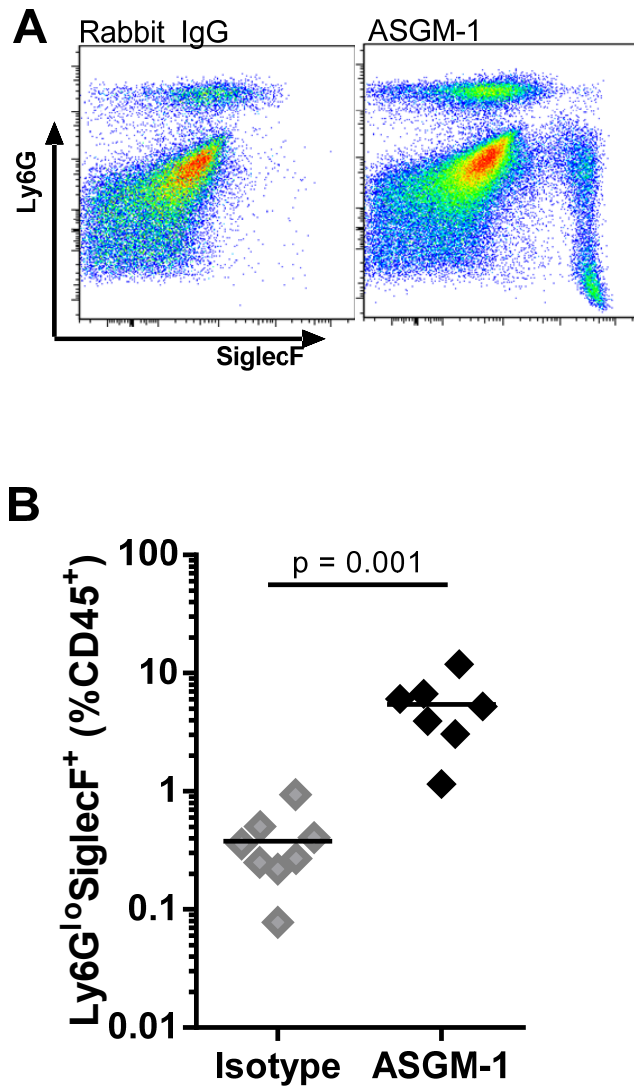
CFs were cultured with either IL4 or both IL4 and magnetically sorted NK cells (1:2 ratio) for 94 hours. Levels of A) CCL11 ($p < 0.001$), B) CXCL9 ($p < 0.001$), C) CXCL10 ($p < 0.001$), D) CCL2 ($p < 0.001$), E) CCL4 ($p < 0.001$), F) CCL5 ($p = 0.002$), G) CXCL1 ($p < 0.001$), and H) IL5 ($p < 0.001$) protein in supernatant were measured by ELISA. Significance by ordinary one-way ANOVA with post-testing by Tukey's multiple comparisons test.

Figure 27. NK cells alter levels of eosinophil-associated chemokines from CFs *in vivo*



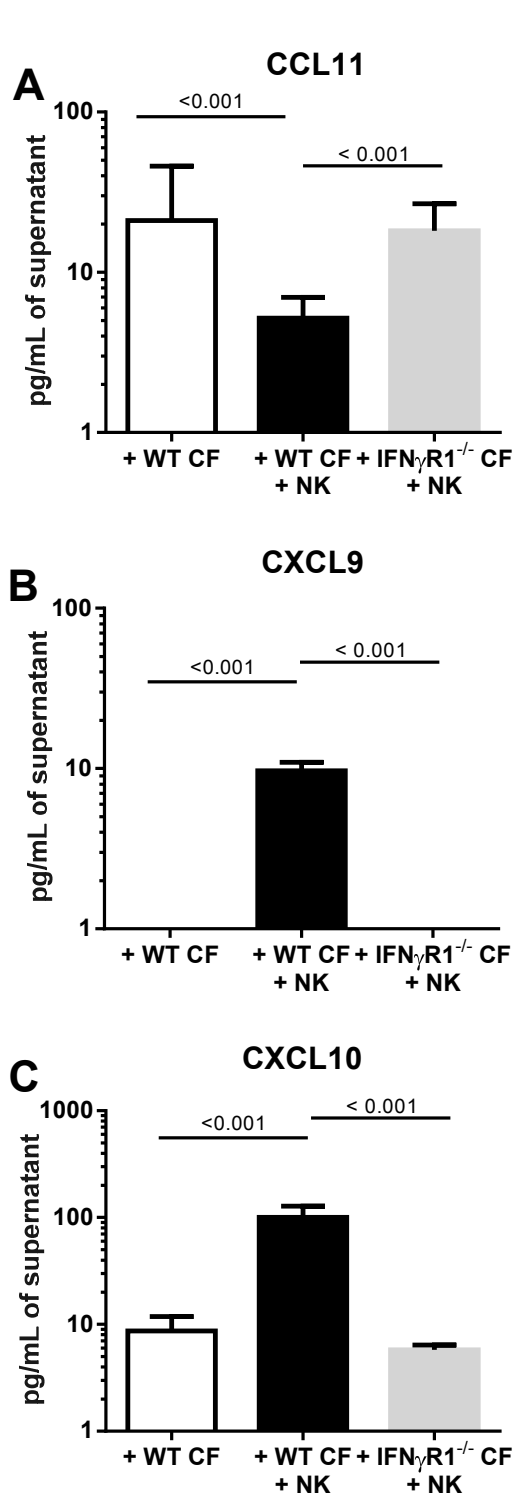
A) *ccl11* ($p=0.064$) and B) *ccl24* ($p=0.020$) mRNA were measured *ex vivo* by qPCR in cardiac fibroblasts isolated at day 21 of disease. Significance calculated by unpaired t-test.

Figure 28. Eotaxins are not required for NK-mediated eosinophil control



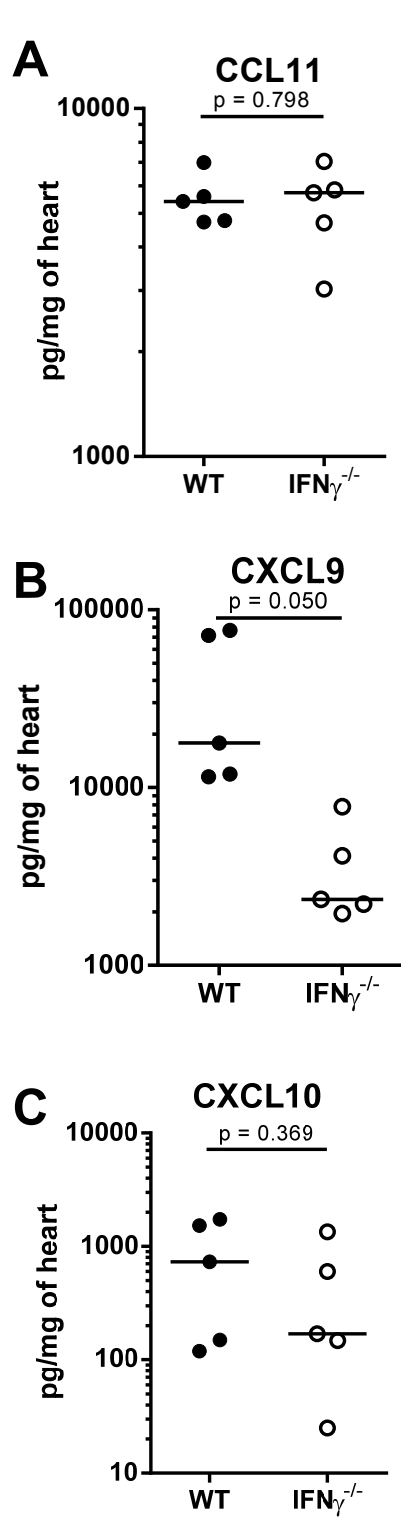
A) Representative bivariate plots of Ly6G versus SiglecF CD45⁺-gated cells at day 21 of EAM in isotype and ASGM-1 antibody treated eotaxin receptor (CCR3)^{-/-} animals. B) Percentage of Ly6G^{lo}SiglecF⁺ eosinophils ($p=0.001$) from total cardiac CD45⁺ cells. Statistics calculated by unpaired t-test

Figure 29. NK cells mediate CCL11, CXCL9 and CXCL10 production from CFs via IFN γ *in vitro*



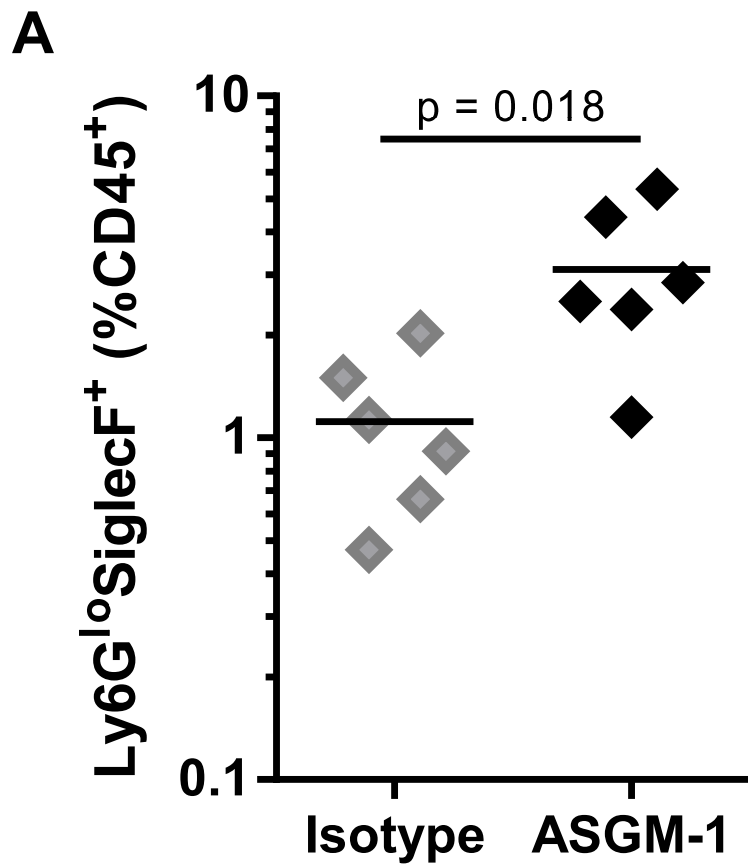
A) CCL11 ($p < 0.001$), B) CXCL9 ($p < 0.001$) and C) CXCL10 ($p < 0.001$) of in the supernatant of magnetically isolated NK cells cultured with either WT or IFN γ R1^{-/-} CFs for 96 hours as measured by ELISA. Significance of C-E by ordinary one-way ANOVA with post-testing by Tukey's multiple comparisons test.

Figure 30. *IFN γ* controls CXCL9 levels during EAM



A) CCL11 ($p=0.798$), B) CXCL9 ($p=0.050$) and C) CXCL10 ($p=0.369$) protein levels at day 21 of EAM in the hearts WT and IFN $\gamma^{-/-}$ mice as measured by LINCO. Statistics calculated by unpaired t-tests.

Figure 31. *IFN γ* is dispensable for NK-mediated control of eosinophil accumulation during EAM



A) Percentage of Ly6G^{lo}SiglecF⁺ eosinophils ($p=0.018$) from total cardiac CD45⁺ cells at day 21 of EAM in isotype and ASGM-1 antibody treated IFN γ ^{-/-} animals.

DISCUSSION

The absence of NK cells from WT mice undergoing myocarditis lead to a specific and significant increase of in the proportion of eosinophils infiltrating the cardiac muscle. While WT isotype control animals had limited numbers of these cells averaging between 0.5 – 2.0% of cardiac CD45⁺ cells, ASGM-1 treatment increased Ly6G^{lo}SiglecF^{hi} numbers ten-fold to approximately 10 – 15% of all infiltrating cells. We hypothesized that this increase in eosinophils was heavily involved in the increase of disease severity seen in the absence of NK cells, as eosinophils are known for their tissue damage potential and role in fibrosis. Furthermore, patients with necrotizing eosinophilic myocarditis have poor prognosis compared to other myocarditis subtypes, indicating that eosinophils are correlated with poor disease outcome in myocarditis.

To determine if the eosinophils seen in the hearts of ASGM-1 animals had the potential for immune modulation and tissue damage, we profiled these cells by surface expression and mRNA levels for activation and maturation markers. Infiltrating eosinophils displayed an activated profile consistent with eosinophils isolated from lungs of mice infected with *Nippostrongylus brasiliensis* with upregulated SiglecF and CD11b²³⁷. As eosinophils aggressively participate in the clearance of *N. brasiliensis* infection, we postulate that similar profiles indicate activate immune modulation by eosinophils of their local environment. Also altered were mRNA levels of MBP and EPX, as mature and activated eosinophils contain granules that were formed during the maturation stages and no longer require synthesis of these proteins²³⁷.

IL6, also upregulated in cardiac eosinophils, has been shown to be induced by IFN γ to co-localize to intercellular granules prior to secretion along with the granule

proteins^{238,239}. Also increased were mRNA levels of TGF β , mirroring the activated population seen in asthmatic airway inflammation models^{237,240}. TGF β also stimulates the development of fibrosis, which along with the deposition of MBP seen in ASGM-1 treated hearts, might be responsible for increase of collagen in NK depleted mice^{241,242}. Therefore, NK depletion likely has limited effects on eosinophils in the periphery, as cardiac eosinophils had distinctly different transcriptional profiles than those found in the spleen.

Eosinophils have recently been shown to regulate muscle repair through the recruitment of fibroblast progenitors in striated muscle by IL4 and IL13¹⁸⁷. It was possible that the increased fibrosis coinciding with the influx of eosinophils during EAM was due to dysregulation of this response mechanism after inflammation-mediated cardiac muscle damage. However, we saw no increase of IL4 and IL13 mRNA in cardiac eosinophils during EAM, signifying this is an unlikely mechanism.

The increase of eosinophils in the heart during EAM in response to NK depletion in no way mirrors the huge influx of eosinophils seen in the tissue of patients with clinical eosinophilic myocarditis or in animal models of eosinophilic myocarditis. In those cases, dysregulation of eosinophils is likely due to more potent regulators of eosinophils, such as Th2-mediated mechanisms as demonstrated in IFN γ /IL17A-deficient mice with eosinophils comprising more than 30% of infiltrating cells after EAM induction. However, we show that even moderate increases in eosinophils can have significant effects on disease outcome. Therefore, the presence of eosinophils in EMB samples, even in moderate numbers, might be considered a risk factor for poor prognosis.

Furthermore, this data opens a new mechanism in the basic regulation of eosinophils. We indicate for the first time, the ability of NK cells *in vivo* to directly suppress eosinophils during inflammation. Th2-associated NK cells have previously been implicated asthma and allergy models to positive regulate eosinophil accumulation through the secretion of IL5. Here, we show that Th1-associated IFN γ producing NK cells can downregulate eosinophil infiltration through communications with cardiac resident cells. Our data support the concept of NK cells as a multifactorial cellular population comprised of heterogeneous and fluid subsets that are highly responsive to their local environment. However, it should be pointed out that NK cells are likely not required in the control of eosinophil trafficking and merely represent one of many mechanisms of regulation.

An alternative hypothesis in the regulation of disease severity by NK cells involves alterations in monocyte populations. NK cells can induce macrophage and dendritic maturation and activation, leading to changes in antigen-specific T cell populations²⁴³. Furthermore, monocytes as the majority of the cells infiltrating the myocardium during EAM have been shown to influence myocarditis severity in EAM^{140,232}.

However, the increased disease severity in the absence of NK cells were not accompanied by changes in levels of CD11b-positive populations or expression of inflammatory monocyte marker Ly6C. Recently, we have published that monocyte control of inflammation in the heart was ultimately due to shifts in the Th cytokine secretion and cell populations, making monocytes the effector population^{36,140,141,244}. Here, despite late changes in Th populations, no changes were observed in the monocyte

compartment, making the case that neither they nor Th2 and Th17 populations were responsible for increased disease severity.

The lack of Th involvement as the ultimate mechanism of increased inflammation and collagen deposition in the absence of NK cells was emphasized by the continued increase of Th2 and Th17-associated cytokines in Δ doubleGATA mice. NK depletion in Δ doubleGATA resulted in increases in total cardiac IL4 and IL6, a surrogate for Th2 and Th17 populations. Despite this increase, no phenotypical changes in disease severity or collagen deposition were seen in the absence of eosinophils during EAM. Therefore, NK cells directly downregulate eosinophilic accumulation along with levels of Th2 and Th17 populations. As the infiltrating cardiac NK cells were largely IFN γ producing, this is consistent with the reported ability of NK cells to downregulate Th2 and Th17 responses through inhibition of anti-specific T cells expressing Ror γ t and GATA3^{245,246}.

We did note limited decreases of basophils due to ASGM-1 treatment corresponding with reports in literature¹²⁷. We expect that this diminution has limited effect as proportions of Th2 cells were elevated after ASGM-1 antibody treatment, the opposite of what would be expected if basophils were involved mechanistically.¹ Mast cells, also implicated in the control of eosinophils in myocarditis, were not different proportionally in the absence of NK cells and likely were not the major regulator of eosinophil infiltration. This was supported by unchanged levels of IL33 and ST2, associated with mast cell activation and ILC2 proliferations. Other than eosinophils, the alterations in basophils were the only significant difference in cardiac infiltrating populations during EAM after NK cell depletion.

We hypothesized that NK cells mediated eosinophilic accumulation in EAM through cardiac resident cells, as our lab recently determined that CFs are a primary mediator in IL17A-mediated EAM²³². Furthermore, NK cell and fibroblast interactions have been well-established. Tumor fibroblasts can interfere with NK cytokine production and cytotoxicity via PGE₂.²⁴⁷ and synoviocytes in RA can express NK-receptor ligands that result in NK activation by direct contact.²⁴⁸ Also, there has been increasing awareness that resident cells play a large role in the determination of cardiac fate during inflammation.²⁴⁹

In our non-exclusive list of chemokines analyzed, both positive and negative mediators of eosinophils were shifted towards a pro-eosinophilic environment in the absence of NK cells. Consistent with our reports of CFs as major mediators of disease, our results demonstrate that NK cells alter eosinophilic chemokine expression *in vivo*, *in vitro*, and *ex vivo* from CFs. Of these, eotaxins, major controllers of eosinophilic trafficking in asthma and allergy, were the most dysregulated²⁵⁰⁻²⁵³. However, chemokines are notorious for functional redundancy and it is difficult to obtain phenotypic evidence for their role in disease^{254,255}. This was supported in EAM by the failure of CCR3^{-/-} and IFN γ ^{-/-} mice to show any changes in eosinophilic accumulation in the absence of NK cells.

CHAPTER V: CONCLUSION

Conclusions

In this study, we demonstrated that the protective qualities of NK cells extend well beyond viral inhibition in myocarditis. By depleting NK cells, but not NKT cells, using treatment with ASGM-1 antibodies, we found that NK cells directly downregulated cardiac inflammation and collagen deposition during EAM, a pathogen-free model of autoimmune myocarditis, by limiting cardiac eosinophil accumulation through interactions with cardiac resident cells (Figure 4).

These data opens the possibility for using NK cells or their products as a biologic therapy for myocarditis. Developments in the treatment for clinical myocarditis is constricted by the opposing needs in the viral and autoimmune components of disease. Ideally, an intervention could be designed that would target both needs simultaneously. Our lab has now shown that NK cells are clearly protective in both viral and autoimmune-mediated driven forms of myocarditis. NK-related therapies are an area of avid cancer research and the same resources could be used to treat autoimmune disorders.²⁵⁸⁻²⁶⁰

Future Directions

Like in all scientific research, the work begun in this dissertation is far from complete. While we were able to clearly define a protective role for NK cells in the pathogenesis of myocarditis and deposition of collagen in the heart, we only showed circumstantial evidence for the specific mechanisms by which NK cells control eosinophilic accumulation. The removal of specific trafficking mediators CCR3 and IFN γ did not restore eosinophils to isotype control levels when NK cells were depleted, signifying our inability to provide hard evidence for the regulation of eosinophils through chemokines. Part of this failure is inherent to the redundant nature of chemokines.

The depletion of NK cells using ASGM-1 is also less than ideal. While we did show that no major alterations in other cell populations occurred, with the exception of basophils, it would be better to confirm these results in an genetically specific NK-deficient BALB/c model, which currently does not exist.

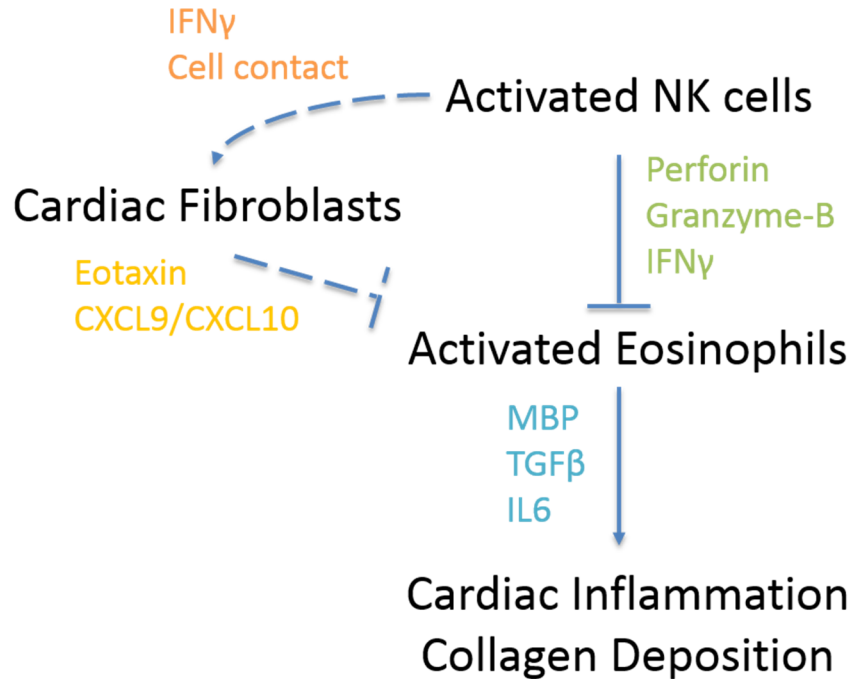
Two major foci remain in the future directions of this research. Firstly, while we investigated the ability of NK cells to modulate disease through resident cells, we did not explore the ability of NK cells to directly interact with eosinophils. In order to do this, we are currently pursuing co-culture experiments between naïve splenic NK cells and mature eosinophils isolated from the blood of IL-5 transgenic animals. As reviewed in the introduction to Chapter III, NK cells have been recently implicated in the activation and subsequent apoptosis of eosinophils *in vitro*. After co-culture, eosinophils will be examined for annexin V and Live/Dead staining in order to determine if the presence of NK cells resulted in increased apoptosis. If this is indicated in the results, it is possible that NK cells in the cardiac environment may directly control eosinophil accumulation by

cell-mediated apoptosis.

Lastly, the pressing question of the role of eosinophils in the pathogenesis of myocarditis and DCMI itself remains. We showed that influx of eosinophils in the heart resulted in overall increased inflammation and collagen deposition, possibly linked to increased TGF β and IL6 secretion and the deposition of MBP in the tissue, but the specifics of eosinophil-mediated damage are unknown in EAM. Certainly, the model of NK depletion in EAM provides a tantalizing glimpse of what pathogenic role eosinophils may play in the heart. Ultimately however, depletion of NK cells only resulted in at most 10% of the cardiac infiltrate being eosinophilic, and thus cannot be considered a model of clinical eosinophilic myocarditis, where the vast majority of cells are eosinophils. We are currently exploring models of eosinophilic myocarditis, either through the use of IL5-transgenic mice or through Th2 deviated myocarditis model that are deficient in both IL17A and IFN γ ²²⁴.

Figures and Tables

Figure 32. Summary of dissertation



Activated NK cells accumulate in the heart during EAM and suppress eosinophilic infiltration through mechanisms that may involve the expression of chemokines from cardiac resident cells. The release of MBP, TGF β , and IL6 from the infiltrating eosinophils into the cardiac environment may be responsible for the resulting increase in cardiac inflammation and fibrosis seen in the absence of NK cells. NK and cardiac fibroblasts interactions *in vitro* led to the release of IFN γ from NK cells in a cell contact-dependent manner. This IFN γ directly modulated the expression of CCL11, CCL24, CXCL9, and CXCL10, chemokines that have direct trafficking effects on eosinophils. Therefore, NK cells may control eosinophil infiltration directly or indirectly through resident cell mediators.

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SUFEY ONG

Curriculum Vitae: August 2014

720 Rutland Avenue
Ross Research Building 648
Baltimore, Maryland 21215
Citizenship: USA

song4@jhmi.edu
(310)308-2188

Education

2014	PhD Department of Molecular Microbiology and Immunology Johns Hopkins School of Public Health, Baltimore, Maryland Advisor(s): Noel R Rose, MD, PhD; Daniela Cihakova, MD, PhD
2008	BA, Public Health University of California - Berkeley, Berkeley, California

Thesis Work

Natural killer (NK) cells control cardiac eosinophilia in experimental autoimmune myocarditis (EAM)

Explores the protective role of NK cells in EAM. Determined that the increased cardiac inflammation, fibrosis, and cardiac dysfunction upon depletion of NK cells in wildtype mice undergoing EAM is directly mediated by increased eosinophil accumulation in the heart due to global changes in both Th profiles and eosinophil-related chemokine production by cardiac resident cells both *in vitro* and *in vivo*.

The autoimmune regulator gene (AIRE) is required for peripheral Th17 responses

Examines the mechanism behind spontaneous candidiasis in clinical patients with genetic defects in the AIRE gene by determining whether Th17 cytokines are AIRE-dependent antigens. Used clonal expansion and single cell sequencing techniques to examine the sequence of T-cell receptors in CD3⁺CD4⁺ cells to identify T-cells reactive against Th17 cytokines.

Relevant Work Experience

2008-2009	Emerging Infectious Disease Fellow Department of Parasitic Disease, Centers for Disease Control and Prevention Atlanta, Georgia Principal Investigator: W. Evan Secor Project: Increased SHIV susceptibility in <i>rhesus macaques</i> infected with schistosomiasis
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Grants and Fellowships

2011-2015	Ruth L. Kirschstein National Research Service Award (NIH) F31 HL112665-01A1: Role of natural killer cells in myocarditis <i>Principal Investigator: SuFey Ong</i>
2010-2011	Otis and Calista Causey Fellow of Immunology
2008-2009	Emerging Infectious Disease Fellowship, American Public Health Laboratories

Publications

Under revision for Circulation Research: **Ong S**, Ligon DL, Barin JG, Wu L, Talor MV, Diny N, Fontes JA, Gebremariam E, Kass DA, Rose NR, Cihakova D. Natural killer cells suppress cardiac inflammation and fibrosis by regulating eosinophilic infiltration.

Wu L, **Ong S**, Talor MV, Barin JG, Baldeviano GC, Kass DA, Bedja D, Zhang H, Sheikh A, Margolick JB, Iwakura Y, Rose NR, Cihakova D. (2014) Cardiac fibroblasts mediate IL17A-driven inflammatory dilated cardiomyopathy. *Journal of Experimental Medicine*. 2014 Jun 16. Epub ahead of print.

Barin JG, Baldeviano GC, Talor MV, Wu L, **Ong S**, Fairweather D, Bedja D, Stickel NR, Fontes JA, Cardamone AB, Zheng D, Gabrielson KL, Rose NR, Cihakova D. (2013). Fatal Eosinophilic Myocarditis Develops in the Absence of IFN- γ and IL-17A. *The Journal of Immunology*, 191(8), 4038-4047.

Barin JG, Baldeviano GC, Talor MV, Wu L, **Ong S**, Quader F, Chen P, Zheng D, Caturegli P, Rose NR, Cihakova D. (2012) Macrophages participate in IL17A-mediated inflammation. *European Journal of Immunology*, 42(3):726-36

Poster Presentations and Scientific Abstracts

Wu L, **Ong S**, Talor MV, Barin JG, Baldeviano GC, Zhang H, et al. (2013, August). IL-17A Gives Rise to Proinflammatory and Profibrotic Monocytes by Inducing GM-CSF Production from Cardiac Fibroblasts. In *Association of Medical Laboratory Immunologists Annual Meeting*. Savannah, Georgia.

Ong S, Ligons, D., Baldeviano, C., Barin, J., Talor, M., Wu, L. et al (2013, June). Natural killer cells suppress cardiac eosinophilia in autoimmune myocarditis. In *Cold Spring Harbor Laboratories. 78th Symposium on Immunity and Tolerance*. CSHL, New York.

Wu L, **Ong S**, Talor MV, Barin JG, Baldeviano GC, Zhang H, et al. (2013, June). IL-17A Gives Rise to Proinflammatory and Profibrotic Monocytes by Inducing GM-CSF Production from Cardiac Fibroblasts. In *Cold Spring Harbor Laboratories. 78th Symposium on Immunity and Tolerance*. CSHL, New York.

Ong S, Ligons, D., Baldeviano, C., Barin, J., Talor, M., Wu, L. et al (2013, April). Natural killer cells suppress cardiac eosinophilia in autoimmune myocarditis. In *Young Investigator's Day, Department of Pathology at Johns Hopkins Medical Institution*. Baltimore, Maryland.

Legault, J. A., Baldeviano, G. C., **Ong S**, Wu, L., Barin, J. G., Talor, M. V. et al (2013). 161: IL6 promotes the progression of experimental autoimmune myocarditis to dilated cardiomyopathy. *Cytokine*, 63(3), 281. San Francisco, California.

Barin, J., Baldeviano, G., Talor, M., Wu, L., **Ong S**, Fairweather, D. et al (2013, May). IL17A and IFN gamma jointly control rapidly fatal eosinophilic myocarditis in mice. In *JOURNAL OF IMMUNOLOGY* (Vol. 190). 9650 ROCKVILLE PIKE, BETHESDA, MD 20814 USA: AMER ASSOC IMMUNOLOGISTS.

Ong S, Ligons, D., Baldeviano, C., Barin, J., Talor, M., Wu, L. et al (2012, May). Natural killer cells suppress cardiac eosinophilia in autoimmune myocarditis. In *JOURNAL OF IMMUNOLOGY* (Vol. 188). 9650 ROCKVILLE PIKE, BETHESDA, MD 20814 USA: AMER ASSOC IMMUNOLOGISTS.

Legault, J., Baldeviano, G., Barin, J., Wu, L., **Ong S**, Talor, et al (2012, May). IL6 is necessary for the progression of experimental autoimmune myocarditis to dilated cardiomyopathy. In *JOURNAL OF IMMUNOLOGY* (Vol. 188). 9650 ROCKVILLE PIKE, BETHESDA, MD 20814 USA: AMER ASSOC IMMUNOLOGISTS.

Baldeviano, G., Barin, J., Wu, L., Talor, M., Ferrer, P., Chen, P, Legault J., **Ong S**, et al. (2011). Eosinophils promote myocarditis and dilated cardiomyopathy by enhancing Th17 responses. In *JOURNAL OF IMMUNOLOGY*, 186, 101-27. 9650 ROCKVILLE PIKE, BETHESDA, MD 20814 USA: AMER ASSOC IMMUNOLOGISTS.

Ong S, Owens, S. M., Chenine, A. L., Ruprecht, R. M., & Secor, W. E. (2009, November). Decreased antiviral antibody responses in shiv1157ip-infected rhesus macaques co-infected with *Schistosoma mansoni*. In *AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE* (Vol. 81, No. 5, pp. 135-136). 8000 WESTPARK DR, STE 130, MCLEAN, VA 22101 USA: AMER SOC TROP MED & HYGIENE.

Teaching Experience

2010-2013	Teaching Assistant Public Health Biology, Johns Hopkins School of Public Health Graduate level class for incoming MPH students comprising of MD and PhD holders. Led student and faculty discussions, graded exams and homework, and answered student questions regarding coursework and logistics.
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Skills and Techniques

12+ color flow cytometry analysis
Advanced and basic mouse skills
Basic histopathology skills
CFA-driven immunization models
ELISA
Grant writing (NIH, AHA, etc.)
Immunofluorescent microscopy
Primary cell isolation and culture
qPCR and RT-PCR
Single cell TCR sequencing